Bovine Lymphocyte Antigen Class II Alleles as Risk Factors for High Somatic Cell Counts in Milk of Lactating Dairy Cows

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ABSTRACT

Lactating Holstein cows (n=1100) from 93 dairy herds in Iowa, Wisconsin, Minnesota, and Illinois were genotyped at the bovine lymphocyte antigen DRB3.2 locus by a genotyping system that used polymerase chain reaction and restriction fragment length polymorphisms. Milk samples were obtained after routine processing at a Dairy Herd Improvement Association facility and returned to the National Animal Disease Center for DNA extraction. Somatic cells were used to classify cows that had acutely elevated SCC (one test of SCC >500,000; group 1), or chronically elevated SCC (three consecutive tests of SCC >300,000 or two consecutive tests of >500,000; group 2), or that were eligible as controls. For each cow in groups 1 and 2, controls were selected that were matched for breed, lactation, herd, and days in lactation (±60 d). A conditional model for stepwise logistic regression was used to determine the relative odds for the 10 alleles with a frequency >3%. No significant associations were observed when the 292 cows in group 2 were compared with their 292 controls. Allele *16 was associated with an increased risk of disease for cows classified with an acute SCC (258 cases and 258 controls). This study has identified DRB3.2*16 as a potential risk factor for acute intramammary infection and established the use of Dairy Herd Improvement Association milk samples as a source of DNA that is useful for genetic epidemiologic studies.

(Key words: mastitis, immunity, major histocompatibility complex, epidemiology)

INTRODUCTION

Diseases that adversely affect milk production by dairy cows, especially diseases involving mammary health (e.g., mastitis), account for the largest reduction in producer income (1, 4, 12, 13, 15, 16, 30, 38). Recent studies (10, 20, 22, 23, 31, 32, 36, 37) have demonstratedheritabilities for several immune parameters and resistance to infectious disease. Although evidence exists for the influence of genetics on the susceptibility of an animal to infectious disease, investigating such relationships is not straightforward. Natural infection rates and genetic heritabilities are low, and large families that are amenable to genetic analysis do not often have adequate records of disease occurrence. Furthermore, the qualitative identification of the disease status occurring in the normal production environment is difficult. Pathogenic challenges of individuals from large families are possible but require financial resources that are not readily available and may not represent normal interactions between environment and genetics. Molecular, epidemiologic studies provide opportunities, with acceptable financial investment, for investigating the association of genetic factors with decreased productivity in large production populations.

One of the difficulties with disease association experiments is determining the disease status of the animal. The SCC have been used by the dairy industry as a measure of intramammary health because SCC are positively correlated with mastitis (29) and because somatic cells have an important role in mammary gland defense (17, 27, 28). Mammary health traits have demonstrated heritabilities (22), and SCC are now used as part of dairy total performance indices of genetic merit. An advantage of using SCC as a measure of individual health is data availability.
The SCC for cows in herds on DHIA testing programs are accumulated approximately once every 30 d throughout lactation. Historical scores are kept for at least six tests (approximately once each month), depending on the dairy records processing center, so that producers can identify animals with continuing mammary health problems.

The bovine major histocompatibility complex (MHC), or bovine leukocyte antigen (BoLA) class II genotyping system, first developed by van Eijk et al. (35), was chosen as a genetic marker system because it required little DNA, was very polymorphic, and had a role in immunity. The genotyping system is based on the polymerase chain reaction and therefore requires little DNA material for analysis. With >30 alleles, the system is the most polymorphic coding sequence described in cattle. The BoLA class II genes play a key role in antigen presentation to T cells (2), and class II positive leukocytes specifically respond to local infusion of Streptococcus uberis in the bovine mammary gland (11). Infusion of the ovine mammary gland with Staphylococcus aureus showed an interaction between MHC class II positive B cells and T cells (18). Also, differential response of bovine CD4+ T cells to Staph. aureus in healthy and infected cows could partially be attributed to less efficient antigen presentation (25). The BoLA gene complex has been investigated for its association with mastitis with varying results (21, 37). Previous serotyping studies of BoLA class I alleles did not find associations with changes of SCC (3).

The purpose of this epidemiologic study was to investigate the association of alleles at the BoLA DRB3.2 locus with acute or chronic elevations of SCC compared with those of matched healthy controls. Genotypes of case and control cows were compared using a conditional logistic regression model for association analysis. This study also demonstrated the broad-based use of milk samples for genotyping of cows.

**MATERIALS AND METHODS**

**Acquisition of Samples and DNA Preparation**

Milk samples were obtained from a commercial DHIA milk testing laboratory (Dairy Lab Services DHIA Laboratory, Dubuque, IA) on three separate dates between January 1 and February 28, 1993. Milk samples were transported to the National Animal Disease Center and stored at 4°C until processed. All milk samples tested contained a preservative (Broad Spectrum Microtabs™; D&F Control Systems, Inc., San Ramon, CA) that is normally used with testing milk samples for SCC, fat, and protein. Samples were processed by spinning 1.5 to 2.0 ml of milk at 11,000 × g in a microcentrifuge for 1 min. The supernatant was removed, and the inside of the tube was swabbed with a cotton tip to remove residual milk fat. Samples were stored at −20°C. Samples were prepared for analysis by the addition of 22 μl of TEN (10 mM Tris and 1 mM EDTA, pH 8.0, and 100 mM NaCl) and 3 μl of 1 M NaOH, vortexed vigorously, and heated to 95°C for 8 min immediately before amplification. The fidelity of DNA obtained from milk was tested using 13 cows from the mastitis research herd of the National Animal Disease Center. Milk was treated with a preservative (Broad Spectrum Microtabs™) and stored at room temperature for 24 h before testing. Genotypes matched those found using DNA obtained from blood samples (data not shown).

**Selection of Affected and Control Animals**

Records for herd analysis and animal matching were obtained from a DHIA record processing center (MidStates Dairy Record Processing Center, Ames, IA). Factors that may influence the frequency of an allele, such as breed, herd, and generation (lactation), as well as factors affecting SCC, such as days in lactation, lactation number, and the extremes in the lactation curve, were controlled by selection of cases and controls. Only Holstein herds were used. The DHIA reports for SCC were obtained for each herd. The DHIA summaries from this processing center report the previous six SCC tests, generally representing the previous 6-mo period. The SCC before a dry period as well as a SCC within 30 d of calving were not used to categorize cows. Two arbitrary categories of elevated SCC were used. Cows were classified as having chronically elevated SCC if they had three consecutive tests of SCC >300,000 or two consecutive tests of >500,000 (group 2). Remaining cows were classified as having an acutely elevated SCC if they had at least one test of SCC >500,000 (group 1). Cows not fitting either classification were considered to be unaffected (control) cows. Each affected cow was matched with the first eligible cow as a control. Matches for affected cows were found in the same herd and were based on lactation number and days in lactation. Lactation categories were first, second, and third or more. Control cows were chosen to be within 60 d in the lactation cycle of the affected cow.
cows. Cows with chronically or acutely elevated SCC without a matching control in the same herd, lactation number, or stage of lactation were not included in the analysis. Finding a matching control cow within a herd was dependent on the size of the herd and the prevalence of elevated SCC within a herd. On average, matching controls were found 75.2% of the time (median of 77.6% and mode of 90%). A matching control was found over 90% of the time for 26% of herds.

BoLA Analysis

The DRB3 alleles were assigned by a hemi-nested method for polymerase chain reaction restriction fragment length polymorphism (35) with the following modifications. The first reaction consisted of 1 μl of prepared milk DNA, 0.5 mM each primer (M0 and M1), 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 50 mM KCl, 1 unit of Taq DNA polymerase (Boehringer Mannheim), and 0.2 mM deoxynucleotide triphosphate solution in a 25-μl volume. The first amplification reaction consisted of an initial denaturation step of 3 min at 94°C, followed by 10 cycles of 94°C for 25 s, 60°C for 30 s, and 72°C for 30 s with a final extension step of 5 min at 72°C. After amplification, 2 μl of the first reaction were transferred to a second hemi-nested reaction. The reaction conditions were the same except that primer M2 was substituted for M1, the deoxynucleotide triphosphate solution was reduced to 0.05 mM, 0.1 μl of [α-32P]dATP [5′ α-33P]triphosphate triethyl ammonium salt, catalog number BF 1001; Amersham Corp., Arlington Heights, IL) was added, and the reaction volume was increased to 45 μl. The amplification reaction consisted of 25 cycles of 94°C for 40 s and 65°C for 30 s with a single, final extension of 72°C for 5 min. All amplification reactions were performed in a thermocycler (GeneAmp PCR System 9600; Perkin Elmer Corp., Norwalk, CT). The amplification reaction product was split into three tubes. Each reaction was incubated with 3.5 units of enzyme and 1.5 μl of reaction buffer at 37°C (HaeIII and RsaI) or 60°C (BstYI) for 1.5 h. After incubation, the BstYI reactions were incubated at 80°C for 5 min. Reactions were electrophoresed on 5% nondenaturing acrylamide (Long Ranger™; AT Biochem, Malvern, PA) gels in 1× TBE (0.089 M Tris base, 0.089 M boric acid, and 0.002 M EDTA, pH 8.0) at 10 W of constant power. Gels were dried and autoradiographed overnight.

Statistical Analysis

Frequency distribution of alleles in groups 1 and 2 was determined. Only alleles with a frequency of >3% were used for analysis. These were alleles DRB3.2*3, *7, *8, *11, *12, *16, *22, *23, *24, and *27. The measure of association between a single putative allele, and disease status was expressed as the relative odds. The dependent variable was the disease state; separate conditional logistic regression models were fit to compare cows with acute elevations of SCC with their controls and for cows with chronic elevations of SCC and their controls. The independent variables were the individual alleles. The same inferences about the estimated relative risk can be obtained from the logistic regression analysis whether the data were obtained from a case-control method (in which the disease states are fixed by design and exposures are assumed to be random) or a cohort method (in which exposures are fixed by design and disease outcomes are random) (5). Because a matched case-control design was used, conditional logistic regression models were fit using EGRET statistical software (SERC Corp., Seattle, WA). Relative odds were obtained from the conditional logistic regression for matched case-control sets (one case and one control) (5). Confidence limits for the univariate odds ratios were derived using the maximum likelihood estimators (5). A positive association of a given allele with disease was indicated by an allele for which the odds ratio was significantly <1; a protective association of an allele was indicated by an odds ratio that was significantly >1.

Enough cows were available to perform subgroup analyses for each lactation of BoLA class II alleles as risk factors for increased SCC. For those subgroup analyses, cows with acute and chronic elevations in SCC were analyzed as a group and compared with their unaffected, matched lactation cohorts. A significance level of P ≤ 0.05 was used for reporting results of analyses.

RESULTS

Cows from 93 herds throughout the upper midwestern US were genotyped. All herds with only Holstein cows were gathered for analysis on the day of sampling. After classification, 1100 cows (550 cases and 550 controls) were genotyped. Use of DHIA milk samples as a source of DNA was largely successful. However, approximately 6% of the samples failed to amplify, and approximately 1% amplified more than one genotype. Repeated attempts at DNA purification from failed samples were unsuccessful. A previous study of 127 Holsteins, using blood-derived DNA, had no individuals that failed genotyping. Therefore, we think that the high level of failed genotyping resulted from sample handling prior to our analysis. Because
of the method used to type the BoLA DRB3.2 locus, certain genotypes could not be discerned into independent alleles (approximately 2.2% of the population). Table 1 presents the allelic frequency found in this population of 1100 Holstein cows. The allele frequencies in this study may not be representative of the entire population represented by the 93 herds because of the selection of specific cows in each herd. Twenty-four previously described alleles were found in this population of cows in addition to 5 new alleles (not previously published). The 6 most frequent alleles accounted for 70.3% of the alleles in the population. The 10 most frequent alleles, which were in a frequency >3%, accounted for 86.1% of the alleles in the population. Nine of these same 10 alleles were among the 10 most frequent alleles in another study (7) and accounted for 84.5% of the alleles in that population of cows.

Cows with chronically elevated SCC represented 12% of all cows found in 93 herds, and the cows with acutely elevated SCC represented 15% of all cows on DHIA test in this population. Table 2 presents the number and classification of cows. Odds ratios and statistical significance are shown in Table 3. To illustrate the proportion of cows in each category by herd, DHIA records were examined on 161 herds that include the 93 herds that were genotyped for this study. As illustrated in Figure 1, 68 and 59% of the 161 herds had 15% or fewer cows categorized as group 1 or group 2, respectively. Over 90% of herds had 20% or fewer cows classified as group 1 and 25% or fewer classified as group 2.

No significant associations were observed when the 292 cows classified as having chronically elevated SCC were compared with their 292 controls. Allele DRB3.2*16 was associated with an increased risk of disease (P ≤ 0.05) for cows classified with an acute SCC (258 cases and 258 controls). Each of the lactation classifications contained alleles associated with increased risk of disease. Allele DRB3.2*8 (P < 0.04) was associated with increased SCC among 324 first lactation cows. Allele DRB3.2*16 (P < 0.003) increased the risk, and allele DRB3.2*22 (P < 0.04) decreased the risk, of a classification of high SCC among 236 second lactation cows. Allele DRB3.2*23 (P < 0.02) was associated with increased SCC for cows in third or greater lactation.

### DISCUSSION

Our study utilized milk that had been obtained after DHIA analysis for the source of DNA from a population of cows. Our protocol used a rapid method for obtaining DNA, greatly reducing the resources necessary to obtain DNA for genetic analysis. Generally, DNA obtained was of a quality high enough to be genotyped on a large scale by PCR RFLP. Some problems did occur with cross-contamination or amplification failure of samples. The amplification failures did not correlate with SCC levels from the DHIA reports. These samples also did not amplify

### TABLE 1. Allelic frequency of bovine lymphocyte antigen class II DRB3 alleles among 1100 Holstein cows.

<table>
<thead>
<tr>
<th>DRB3.2 Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>14.29</td>
</tr>
<tr>
<td>24</td>
<td>14.29</td>
</tr>
<tr>
<td>8</td>
<td>14.1</td>
</tr>
<tr>
<td>16</td>
<td>10.01</td>
</tr>
<tr>
<td>23</td>
<td>9.1</td>
</tr>
<tr>
<td>11</td>
<td>8.51</td>
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<td>7</td>
<td>5.32</td>
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<td>3</td>
<td>3.96</td>
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<tr>
<td>27</td>
<td>3.69</td>
</tr>
<tr>
<td>12</td>
<td>3.14</td>
</tr>
<tr>
<td>26</td>
<td>2.32</td>
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<tr>
<td>10</td>
<td>1.36</td>
</tr>
<tr>
<td>28</td>
<td>1.36</td>
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<tr>
<td>21</td>
<td>0.96</td>
</tr>
<tr>
<td>15</td>
<td>0.64</td>
</tr>
<tr>
<td>18</td>
<td>0.64</td>
</tr>
<tr>
<td>9</td>
<td>0.45</td>
</tr>
<tr>
<td>20</td>
<td>0.32</td>
</tr>
<tr>
<td>2</td>
<td>0.27</td>
</tr>
<tr>
<td>13</td>
<td>0.27</td>
</tr>
<tr>
<td>14</td>
<td>0.27</td>
</tr>
<tr>
<td>25</td>
<td>0.23</td>
</tr>
<tr>
<td>17</td>
<td>0.14</td>
</tr>
<tr>
<td>1</td>
<td>0.09</td>
</tr>
<tr>
<td>Uninformative</td>
<td>4.2</td>
</tr>
<tr>
<td>Undescribed</td>
<td>0.1</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
</tr>
</tbody>
</table>

1 Genotypes that could not be distinguished into specific alleles.
2 New alleles identifiable but not described by van Eijk et al. (35).
other PCR primer sets, and no other methods for extracting DNA were successful. The cross-contamination may have occurred because milk left in the milking machines or collection jars may have had enough somatic cells to cause occasional amplification of more than one genotype because of the sensitivity of PCR. This problem might be alleviated by informing DHIA supervisors of the requirements for collection of milk samples to be used for DNA analysis. Possibly, the 1% of samples that gave multiple genotypes were the result of fraternal twins. Published reports of twinning rates for cattle range from 0.9 to >5% of births, depending on the age of the cow (9). Population means suggest an overall twinning rate of 2 to 3% as being quite possible (14), although rates of fraternal twinning would be less.

Our method utilized commercial production populations of dairy cows as a source of DNA samples, and DHIA was the source for health records. Genetic epidemiological studies of health in the production populations have previously been used to investigate the associations of interferon genotypes with the severity of bovine herpesvirus 1 infection (26), BoLA class I serotypes with chronic posterior spinal paresis, and BoLA class II alleles with bovine leukemia virus (6, 34), among others. The current findings should be viewed as preliminary until verified independently.

Associations of disease among farm animals with MHC alleles have been reviewed (24). Although these populations are often statistically difficult to analyze, genetic epidemiological studies are necessary to determine whether further, more directed studies are needed. We chose to categorize the study population into affected and unaffected cows. We were not as interested in the reduction of the baseline SCC as much as the use of SCC to signal an intramammary infection. However, after examination of a number of herds for SCC, we chose to categorize the affected cows into classes of acutely and chronically elevated SCC. We also tried to reduce variation of the allelic frequency and of SCC by matching controls for breed, herd, days in lactation, and lactation number.

### Table 3. Odds ratios and 95% confidence interval (CI).

<table>
<thead>
<tr>
<th>Classification</th>
<th>DRB3.2 Allele</th>
<th>P</th>
<th>Odds ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronically elevated</td>
<td>0</td>
<td>0.049</td>
<td>0.640</td>
<td>0.411–0.997</td>
</tr>
<tr>
<td>Acutely elevated</td>
<td>16</td>
<td>0.031</td>
<td>0.351</td>
<td>0.295–0.957</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lactation</th>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>0.001</td>
<td>0.333</td>
<td>0.163–0.682</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>0.037</td>
<td>1.875</td>
<td>1.022–3.440</td>
</tr>
<tr>
<td>≥3</td>
<td>22</td>
<td>0.019</td>
<td>0.558</td>
<td>0.339–0.920</td>
</tr>
</tbody>
</table>

*Alleles were associated with an increased likelihood of disease with an odds ratio <1. A protective effect is seen with an odds ratio >1.*
We have detected associations between alleles at the BoLA class II DRB3.2 locus and acute increases in SCC in a retrospective study using production populations. No associations were observed between chronically infected cows and BoLA DRB3.2 alleles. Because our analysis of disease did not distinguish among the events that cause increases in SCC, the influence of the BoLA alleles on the immune response might differ significantly among stimuli, leading to the two disease classifications. For example, Staph. aureus and Strep. agalactiae tend to cause more sustained increases in SCC, but Escherichia coli usually causes immediate increases in SCC that may quickly subside. However, some organisms can cause acute disease in one cow but chronic disease in another, a difference that might certainly have a genetic basis. Alternatively, chronically infected cows may reflect a large environmental influence on the phenotype through poor management of intramammary health before, during, or after infection.

Allelic associations were detected for cows categorized by lactation number. However, because of the small sample size, analysis of lactation number by acute and chronic subgroups lacked adequate statistical power. Consequently, cows with acutely or chronically increased SCC were grouped for analysis of BoLA class II alleles as risk factors. The same alleles were not shown to be risk factors for all lactations. Only allele DRB3.2*16 was determined to be a risk factor for acute increases in SCC and increases (both chronic and acute) of SCC for second lactation cows. Surprisingly, different alleles for each lactation number were associated with elevated SCC. Factors causing these different associations with parity might include changes that occur throughout the lifetime of lactating cows (infections during dry periods for second lactation cows or changes in udder conformation as a cow ages) and might influence the microenvironment of the mammary gland. Alternatively, one of the most important concerns is the culling of cows that are susceptible to infection. Some of the first lactation cows that are chronically infected may not survive in the productive population until their second lactation. To analyze accurately the impact of these alleles and their protective (or susceptible) effects, a longitudinal study from calving to final lactation would be very useful. Little is known about the dynamics of allelic frequency at the BoLA locus or the possible effects of selection for high producing and healthy cows on gene frequency. Reports have indicated changes in allelic frequency over time (33).

In a companion report (7) of a research herd of Holstein cows, an allelic substitution model found that alleles DRB3.2*8, *16, *22, and *28 were associated with elevated somatic cell scores. Cows carrying alleles DRB3.2*16 and *24 were more susceptible to intramammary infections caused by major pathogens, and alleles DRB3.2*11 and *23 were associated with increased resistance to clinical mastitis and intramammary infections caused by major pathogens (7). Thus, by consensus, cows carrying alleles DRB3.2*8 and *16 appeared to be more susceptible to mastitis, and cows with alleles DRB3.2*11 and *23 appeared to be more resistant to mastitis or its effects (e.g., elevated SCC). Lewin (19) reported that alleles DRB3.2*8, *16, *22, and *24 are associated with susceptibility to persistent lymphocytosis caused by bovine leukemia virus, and alleles DRB3.2*11, *23, and *28 are associated with resistance to the persistent lymphocytosis effect of infection by the bovine leukemia virus. The explanation for the effects of susceptibility and resistance of these alleles on viral disease symptoms, mastitis incidence, and susceptibility values are not known, but, based upon this current report and two independent analyses of the effects of the alleles at the DRB3.2 locus on disease (8, 19), DRB3.2*8 and *16 appeared to be associated with susceptibility, and DRB3.2*11 and *23 appeared to be associated with resistance to selected diseases of cattle. Additional associations with disease resistance may emerge as additional populations of cattle are analyzed under various management conditions.

This study looked at a small portion of the production environment. The associations found may be applicable only to the time and population analyzed or may have been due to chance alone. The use of milk samples as a source of DNA dramatically reduces the resources necessary for genotyping cattle and may allow longitudinal epidemiological studies to determine the significance of BoLA class II alleles throughout the productive lifetime of the cow. These results suggest that production populations are useful for the investigation of interactions of alleles and phenotypes.

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7 Reference deleted in proof.


