Major histocompatibility complex recombinant *R*13 antibody response against bovine red blood cells[1]

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ABSTRACT Recombination within the chicken major histocompatibility complex (**MHC**) has enabled more precise identification of genes controlling immune responses. Chicken MHC genes include *B*F, MHC class I; *B*L, MHC class II; and *B*G, MHC class IV that are closely linked on chromosome 16. A new recombination occurred during the 10th backcross generation to develop congenic lines on the inbred Line UCD 003 (*B*17*B*17) background. Recombinant *R*13 (*BF*17-*BG*23) was found in a single male chick from the Line 003.R1 (*BF*24-*BG*23) backcross. An additional backcross of this male to Line UCD 003 females increased the number of R13 individuals. Two trials tested this new recombinant for antibody production against the T cell–dependent antigen, bovine red blood cells. Fifty-one progeny segregating for *R*13*R*13 (n = 10), *R*13*B*17 (n = 26), and *B*17*B*17 (n = 15) genotypes were produced by a single *R*13*B*17 male mated to 5 *R*13*B*17 dams. One milliliter of 2.5% bovine red blood cell was injected intravenously into all genotypes at 4 and 11 wk of age to stimulate primary and secondary immune responses, respectively. Blood samples were collected 7 d after injection. Serum total and mercaptoethanol-resistant antibodies against bovine red blood cell were measured by microtiter methods. The least squares ANOVA used to evaluate all antibody titers included trial and B genotype as main effects. Significant means were separated by Fisher's protected least significant difference at $P < 0.05$. *R*13*R*13 chickens had significantly lower primary total and mercaptoethanol-resistant antibodies than did the *R*13*B*17 and *B*17*B*17 genotypes. Secondary total and mercaptoethanol-resistant antibodies were significantly lower in *R*13*R*13 chickens than in *R*13*B*17 but not *B*17*B*17 chickens. Gene differences generated through recombination impacted the antibody response of *R*13 compared with *B*17. Secondary antibody titers were not substantially higher than the primary titers suggesting that the memory response had waned in the 7-wk interval between injections. Overall, the results suggest that the lower antibody response in *R*13*R*13 homozygotes may be caused by recombination affecting a region that contributes to higher antibody response.

Key words: immunity, recombination, antibody, MHC

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INTRODUCTION

Chicken immune responses are controlled by genes within the chicken major (*B*) histocompatibility complex (**MHC**). Class I, class II, and class IV molecules are encoded by the specific chicken MHC *B*F, *B*L, and *B*G genes, respectively (Pink et al., 1977; Kaufman et al., 1999; Miller and Taylor, 2016). Close proximity of the *B*F and *B*L genes hinders genetic recombination (Skjodt et al., 1985). However, there is infrequent but measurable recombination between the *BF/BL* genes and *BG* genes (Briles and Briles, 1982; Skjodt et al., 1985; Miller and Taylor, 2016). The outcome of diseases caused by various pathogens including those from bacteria, viruses, and parasites exhibits MHC control (Taylor, 2004; Miller and Taylor, 2016).

Responses to nonpathogenic antigens such as synthetic polypeptides and allogeneic red blood cells are also controlled by variation within the MHC. Two independent experiments found changes in MHC haplotype frequencies over multiple generations of selection for

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high or low antibody response after sheep red blood cell (**SRBC**) immunizations. Birds selected for high antibody against SRBC were fixed for *B*21 (Martin et al., 1990; Pinard et al. 1993; Dorshorst et al. 2011). The 2 selections used different birds, antigen doses, and routes of antigen administration, yet the same *B*21 haplotype produced high antibody response. Haplotype *B*21 also demonstrated significant resistance to Marek's disease (**MD**) (Briles et al., 1977; Miller and Taylor, 2016). The low antibody selection fixed *B*13 in one line (Martin et al., 1990; Dorshorst et al. 2011) and *B*14 in another line (Pinard et al. 1993).

Congenic lines are developed by introducing an alternate allele, called the differential gene, into a highly inbred line. The source of the differential gene may be an inbred or outbred stock. Multiple backcrosses to the inbred line with selection of the desired genetic variant are used to increase the amount of the inbred background genome and thereby reduce the impact of genes other than the specific selected gene (Miller and Taylor, 2016). Chicken lines congenic for MHC genes have been produced on several genetic backgrounds. Bacon et al. (1987) developed congenic lines using the inbred Line $15I_5$ background. Higher antibody responses against SRBC were found in Lines 15.C-*12* (*B*12*B*12) and 15.N-*21* (*B*21*B*21) than those in Line 15.P-*13* (*B*13*B*13). In congenic lines developed on the inbred Line 6_1 background, total anti-SRBC antibody was significantly higher in 4- and 7-wk-old Line 6.15-*5* (*B*5*B*5) than in Line 6.6-*2* (*B*2*B*2) (Dix and Taylor, 1996).

Major histocompatibility complex recombinants, discovered by Elwood Briles at Northern Illinois University (Briles et al., 1982), were introduced into the inbred Line UCD 003 background to develop MHC congenic lines. Ten backcross generations to UCD 003 stock produced 6 lines, each of which carried an MHC recombinant that arose from a unique recombinational event (Miller et al., 2004; Schulten et al., 2007; 2009).

Line UCD 003 background MHC recombinants Lines 003.*R*5 (*B*F21-*B*G19) and 003.*R*6 (*B*F21-*B*G23) had higher antibody against SRBC than the levels found in 4 recombinants that did not have *B*F21 (Schulten et al., 2007). The outcome of Rous sarcoma virus tumors also differed among 5 of these congenic lines. Lines 003.*R*2 (*B*F2-*B*G23), 003.*R*5, and 003.*R*6 had lower tumor growth than did lines 003.*R*1 (*B*F24-*B*G23) and 003.*R*4 (*B*F2-*B*G23) (Schulten et al., 2009). The response difference between 003.*R*2 and 003.*R*4 had special interest because these 2 recombinants were similar by serology but originated from independent recombination events. Molecular analysis revealed that 003.*R*4 BG1 region had a 255-bp insert in the 3′ untranslated region (**UTR**) that was not present in 003.*R*2 (Goto et al., 2009). These 2 lines also had different responses against MD. The tumor growth difference indicated the BG1 gene region as a candidate for Rous sarcoma virus and MD response via an unknown mechanism (Miller and Taylor, 2016).

The objective of this study was to examine antibody production against BRBC in segregating haplotype combinations of *R*13 and *B*F17. The new MHC recombinant, *R*13, contains *B*F17 and *B*G23 on the Line UCD 003 genetic background. Genetic identity, calculated as >99.9% after 11 backcross generations, minimized background genes effects in the test birds.

MATERIALS AND METHODS

Stock

Congenic lines containing 6 different MHC recombinants were produced as described. Briefly, each of 6 *B* complex recombinants was crossed to inbred line UCD 003 (*B*17*B*17). Six congenic lines with 99% background genome identity resulted from 10 backcrosses to the inbred line (Schulten et al., 2007).

A new recombinant *B* haplotype, designated as R13 (*B*17r1, *B*F17-*B*G23), was found in the 10th backcross generation for R1 (*B*24r1, *B*F24-*B*G23). Because the *R*13 recombinant was discovered in a single male, 1 additional backcross (11th generation) was made to the background Line UCD 003 to increase the number of individuals carrying *R*13. Two separate experiments used heterozygous *R*13*B*17 chickens from the 11th backcross generation as parents. A single *R*13*B*17 sire was mated to 5 *R*13*B*17 dams to produce the experimental progeny that segregated for *R*13*R*13, *R*13*B*17, and *B*17*B*17 MHC genotypes. The genetic background was UCD003, as these were derived from the founding *R*13 individual of the 10th backcross generation.

Eggs were incubated and hatched under standard conditions. At hatch, chicks were wing banded for identification and vaccinated against MD. Newcastle–bronchitis vaccine was administered at 10 d of age. Housing consisted of heated brooder batteries for the first 6 wk followed by grower cages for the remainder of the study. Antibiotic-free food and water were provided for ad libitum consumption throughout the study. All procedures were approved by the appropriate institutional animal care and use committee.

Haplotype Identification

Hemagglutination assays with antisera specific for parental *B* haplotypes were used to test the *B* haplotypes of all individuals as described (Briles and Briles, 1982; LePage et al., 2000; Schulten et al., 2007). From each bird, 500 μL blood was collected into anticoagulant solution (68 μmol/L sodium citrate/72 μmol/L sodium chloride). Samples were shipped on ice, overnight to Northern Illinois University. Fifty microliters of 2% washed erythrocyte in 0.9% saline solution was added to 100 μL of the diluted *B* haplotype–specific antisera of interest in 10 × 77 mm tubes. These solutions were incubated at room temperature for 2 h and then overnight at 3°C. Solutions were then resuspended, incubated at room temperature for 1 h, and read for the presence of agglutination.

Antigen Inoculation

Bovine red blood cells (**BRBC**), a T cell–dependent antigen (McArthur et al., 1973), were collected in Alsever's solution. The BRBC were washed thrice in 0.9% sterile NaCl solution. The BRBC were then diluted to 2.5% (vol/vol) solution in 0.9% sterile NaCl. Two trials were conducted using a total of 51 progeny hatched from the *R*13*B*17 heterozygous parents' mating. At 4 wk of age, blood samples were taken from each chick before BRBC injection to determine if any cross-reacting antibody preexisted. Chicks then received brachial intravenous injections with 1 mL of 2.5% washed BRBC solution. Blood was collected 7 d after antigen injection for primary antibody response measurements. The serum, recovered after blood clotting and centrifugation, was stored at −20 C. Chicks were injected a second time with the 2.5% BRBC solution at 11 wk of age for secondary antibody response measurements. Blood samples for the secondary response were taken 7 d after antigen injection.

Antibody Titration

Serum complement was inactivated in all samples by incubation at 56°C for 1.0 h. Primary and secondary antibody responses were measured in serum samples taken 7 d after antigen inoculation at 4 and 11 wk of age. Total antibody measurements used the established microtiter procedure (Wegman and Smithies, 1966; LePage et al., 2000; Schulten et al., 2007). Mercaptoethanol (**ME**)-resistant antibody (IgG) against BRBC was evaluated using the technique developed by Yamamoto and Glick (1982) as adapted (LePage et al., 2000; Schulten et al., 2007). Titers were expressed as $\log_2$ of the reciprocal of the highest dilution exhibiting visible agglutination.

Statistical Analysis

Least squares ANOVA was used to analyze the antibody titers against BRBC. In both primary and secondary responses, total and ME-resistant antibody was examined. Trial and *B* genotype were the main effects in the statistical model. Fisher's protected least significant difference at $P < 0.05$ was used to separate significant means.

RESULTS AND DISCUSSION

Recombination was detected in a male produced in the 10th backcross of *R*1 to Line UCD 003. The *R*1 contains *B*F24 and *B*G23 and thus should react to both types of antisera in tests to identify MHC types. An anomaly was detected when 1 male, expected to be *R*1*B*17, reacted with antisera specific for *B*G23 but not *B*F24. Further analyses using a panel of antisera revealed reactions with *B*F17 and *B*G23 antisera identifying that male as a new recombinant, designated *R*13 (Table 1). The *R*1 and *B*17 types in the sire had recombined at an unknown crossover point to produce R13 (*B*F17-*B*G23). This single *R*13 male, heterozygous with *B*17 in the 10th backcross, underwent an additional backcross to the UCD 003 background line in the 11th generation to increase the number of individuals carrying the new recombinant.

Table 1. Origin of major histocompatibility (*B*) complex recombinant *R*13. A *B* complex recombinant *R*1*B*17 sire backcrossed in the 10th generation (BC10) to inbred white Leghorn Line UCD 003 (*B*17*B*17) dams produced *R*1*B*17 and *B*17*B*17 progeny as expected. A single male that did not react to the B24 antisera was identified as a recombinant between R1 and *B*17, designated R13.

Individual	Genotype	Component Haplotypes	B-G	B-L	B-F
Sire (BC10)	R1B17	*R*1	23	24	24
		*B*17	17	17	17
Dams UCD 003	*B*17*B*17	*B*17	17	17	17
		*B*17	17	17	17
Recombinant male chick	*R*13*B*17	*R*13	23	17	17
		*B*17	17	17	17

A total of 51 chicks, differing only for their MHC haplotype, were produced from a mating of heterozygous *R*13/*B*17 parents from the 11th backcross generation. The genotypes *R*13*R*13 (n = 10), *R*13*B*17 (n = 26), and *B*17*B*17 (n = 15), defined by serology, segregated in the expected 1:2:1 ratio ($X^2 = 1.0$, 2 *df*, $P = 0.606$).

Animals were injected with 1 mL of 2.5% BRBC at 4 wk of age to induce a primary antibody response. Blood samples were taken from the chicks 7 d after injection for primary immune response antibody evaluation. Total and ME-resistant (IgG) antibodies against BRBC were measured on a $\log_2$ scale using microtiter methods. No preexisting antibody against BRBC was found in any of the test birds. The higher total primary antibody titers in both *B*17*B*17 and *R*13*B*17 genotypes differed significantly ($P < 0.05$) from the titers of *R*13*R*13 chickens (Figure 1). The recombination breakpoint, which produced *R*13, may have damaged one or more genes that impact antibody production. A dominant effect of *B*17 over *R*13 explained the higher antibody in heterozygous *R*13*B*17 birds.

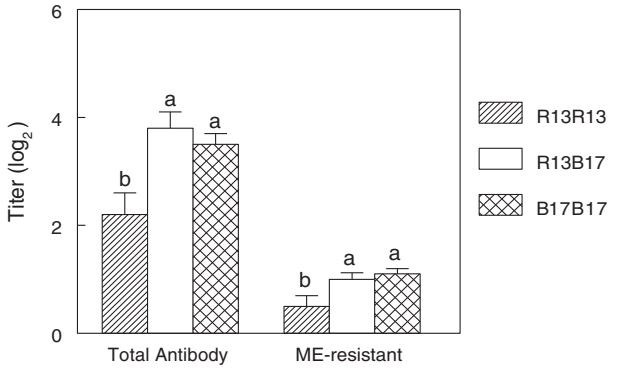

Figure 1. Mean (±SEM) total and mercaptoethanol (ME)-resistant primary antibody titers for 4-wk-old *R*13*R*13, *R*13*B*17, and *B*17*B*17 congenic chicks 7 d after intravenous inoculation with 2.5% BRBC solution. Bars without a common letter differ significantly ($P < 0.05$).

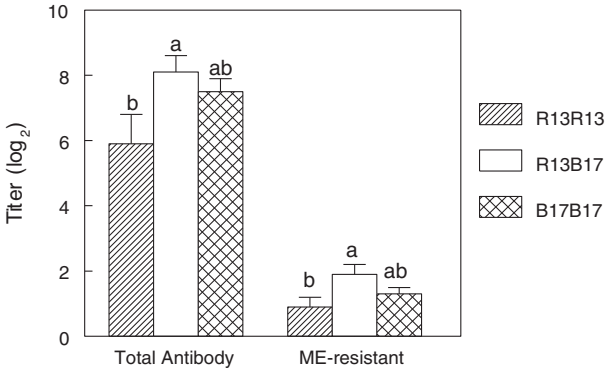

Figure 2. Mean (±SEM) total and mercaptoethanol (ME)-resistant secondary antibody titers for 11-wk-old *R*13*R*13, *R*13*B*17, and *B*17*B*17 congenic chicks 7 d after intravenous inoculation with 2.5% BRBC solution. Bars without a common letter differ significantly ($P < 0.05$).

The titers were similar to previous results found in 6 lines congenic for MHC recombinants (Schulten et al., 2007). The lines in the former study were immunized with 2.5% SRBC, whereas the current work used the same dose of BRBC as the antigen. Among the 6 congenic lines in the former study, 003.R5 and 003.R6, had the highest titers. Both of these genotypes have *B*F21, which is associated with higher antibody responses in multiple lines (Martin et al., 1990; Pinard et al. 1993; Dorshorst et al. 2011). Each MHC recombinant, contained in the congenic lines, arose from an independent event, meaning that the precise chromosomal breakpoint and the genes contained therein are different. One example is found in recombinants R2 and R4. A 225-bp insert in the B-G1 3′ UTR is found in R4 but is absent in R2 (Goto et al., 2009). Furthermore, congenic line 003.R2 has a better response against MD (Schat et al., 1994; Goto et al., 2009) and the Rous sarcoma virus (Schulten et al., 2009) compared with 003.R4. The insert into the B-G1 3′ UTR impacted these responses in an unknown manner.

The new R13 recombinant has *B*F17 and *B*G23 compared with the *B*17 haplotype that has *B*F17 and *B*G17 (Table 1). The current data suggest that *B*G23 has a negative effect on antibody production in the homozygous state. No *B*G region effect on primary antibody titers has been observed in previous studies. Schulten et al. (2007) found that the congenic MHC recombinant lines 003.R5 (*B*21r5, *B*F21-*B*G19) and 003.R6 (*B*21r6, *B*F21-*B*G23), whose *B*G region differed had similar primary antibody titers but different secondary titers against SRBC. On the other hand, MHC recombinant lines 003.R2 (*B*2r1, *B*F2-*B*G23) and 003.R4 (*B*2r3, *B*F2-*B*G23), which differ by the 225-bp insert, had similar primary and secondary anti-SRBC responses (Schulten et al. 2007). The current results coupled with the previous study warrant further examination of possible *B*G effects.

Two Ig classes, IgM and IgG, comprise the total agglutinating antibody response. Antibody production begins with IgM followed by a switch to IgG. Treatment with ME degrades IgM leaving the ME-resistant IgG. The primary antibody response had low IgG titers as expected. The ME-resistant antibody results followed those of total antibody in that the titers of genotypes *B*17*B*17 and *R*13*B*17 were significantly higher ($P < 0.05$) than *R*13*R*13 birds (Figure 1).

The antibody studies of Schulten et al. (2007) and the current work used 2.5% SRBC or BRBC, respectively, in congenic lines with MHC recombinants. The 2.5% RBC dose may have exceeded the threshold for response in all genotypes. A higher or lower antigen concentration might produce different results. The selection experiments for high or low antibody to SRBC (Siegel and Gross, 1980) used 0.1 mL of 0.25% SRBC as the antigen dose. This process produced a different response threshold in high or low antibody lines (Boa-Amponsem et al., 2000). A lower antigen dose in *R*13*R*13, *R*13*B*17, and *B*17*B*17 genotypes may have revealed a different response threshold among the genotypes.

At 11 wk of age, the chicks were injected with 1 mL of 2.5% BRBC the second time to evoke a secondary immune response. Blood samples were taken from the chicks 7 d after injection for total secondary and (ME-)resistant antibody response evaluation. Analysis of total secondary antibody revealed a significant difference between the highest titer genotype *R*13*B*17 and the lowest titer genotype *R*13*R*13. The intermediate total secondary antibody titer of genotype *B*17*B*17 had no statistical difference from either *R*13*B*17 or *R*13*R*13 (Figure 2).

The total secondary titers in *R*13*R*13 birds were lower than those found in 6 congenic lines carrying MHC recombinants (Schulten et al., 2007). Genotypes *R*13*B*17 and *B*17*B*17 had titers similar to the previous results. A genotype difference is the heterozygous combination of *B*F17 and *B*G23. The lower secondary titer may be a product of this combination, in part due to the *B*G effect or the recombination breakpoint may have altered a gene involved with the antibody response. Schulten et al. (2007) injected SRBC, whereas the present study used BRBC, yet both studies had secondary titers of comparable magnitude. Attributing the lower secondary antibody titer in a single genotype to antigenic differences between SRBC and BRBC seems improbable.

The secondary ME-resistant antibody (IgG) titers were consistent with the secondary total titers. Chicks of the genotype *R*13/*B*17 had the highest titer, which differed significantly from genotype *R*13*R*13. The titer of *B*17*B*17 birds had no significant difference from either *R*13/*B*17 or *R*13*R*13 (Figure 2). Higher amounts of IgG would have been expected in a secondary response. In fact, the IgG titers were similar to those found in the primary response. This unusual feature may be attributed to a secondary response that waned before 11 wk or to an overall lower secondary response in these genotypes. Neither possibility may be confirmed nor refuted from the current data.

In summary, chickens congenic for a new MHC recombinant, R13 (*BF*17-*BG*23), had differential antibody response against BRBC. Birds having *R*13*R*13 had lower response that differed significantly from *R*13/*B*17 and *B*17*B*17 in the total primary antibody but only from *R*13/*B*17 in the total secondary antibody. Further

studies of this new recombinant are needed to refine our understanding of MHC genes and the impact of their variation on antibody responses.

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