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Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

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Antibody Response Against Sheep Red Blood Cells in Lines Congenic for Major Histocompatibility (*B*) Complex Recombinants[1]

E.S. Schulten[2], L.M. Yates[3] and R.L. Taylor, Jr.[2]
[2]Department of Animal and Nutritional Sciences, University of New Hampshire, Durham, NH 03824, USA
[3]Department of Biological Sciences, Northern Illinois University, DeKalb, IL 60115, USA

Abstract: Six congenic lines containing *B* complex recombinants R1 = *B-F/B-L24, B-G23;* R2 = *B-F/B-L2, B-G23;* R3 = *B-F/B-L2, B-G23;* R4 = *B-F/B-L2, B-G23;* R5 = *B-F/B-L21, B-G19;* and R6R6 = *B-F/B-L21, B-G23* were tested individually for antibody response against SRBC. R2, R3 and R4 arose from independent recombination events but are serologically identical. Each *B* complex recombinant was crossed to inbred Line UCD 003 (*B*17*B*17). After ten backcross generations to the inbred line, *B* complex heterozyogtes were mated to produce recombinant homozygous lines having 99.9% background gene uniformity. Birds of each line were injected intravenously with 1 mL of 2.5% SRBC at four and 11 weeks of age to induce primary and secondary antibody responses, respectively. Blood samples were collected 7 days post-injection. Microtiter methods were used to assay total anti-SRBC and mercaptoethanol-resistant (MER) serum antibody. All antibody titers were evaluated by least squares ANOVA with hatch and B recombinant genotype as main effects. Fisher's protected LSD was used to evaluate significant means. Genotypes R5R5 and R6R6 had significantly higher primary total antibody titer to SRBC compared with R1R1, R2R2, R3R3 and R4R4. Both R5 and R6 have BF21, a haplotype known for high antibody response to SRBC. Primary MER antibody response did not differ significantly among the genotypes. The total and ME-resistant secondary antibody titers of R5R5 chickens were significantly higher than the other five recombinants. These results indicate that congenic lines carrying six *B* complex recombinants differ in their primary and secondary antibody responses to SRBC.

Key words: *B* complex, SRBC, congenic, antibody, immune response

Introduction

Effective immune responses protect organisms against disease insults. Innate immunity is nonspecific, occurs immediately and lacks memory whereas adaptive immunity is specific, occurs following a lag phase and shows memory through a greater, more rapid response to subsequent antigen exposure. Antibodies represent the humoral component of adaptive immunity. The exogenous antigen presentation pathway stimulates an antibody response to a foreign antigen through coordinated, sequential events. An antigen presentation cell (APC) phagocytizes and degrades antigens into component peptides. Component antigenic peptide:MHC class II complexes appear on the APC surface for T helper (T_h) cell recognition. This interaction activates the T_h cell, which then stimulates B lymphocytes to produce immunoglobulins (Ig) specific for the immunizing antigen. These protein molecules neutralize toxins, opsonize pathogens for phagocyte recognition and activate complement (Parham, 2000).

Fundamental properties for immunogens to stimulate an antibody response are foreignness, molecular size, chemical complexity and degradability. Immunogen dose, route of administration and the genetic constitution of the immunized animal are important host factors that influence the antibody response (Parham, 2000). Divergent selection experiments show that multiple genes affect antibody responses. Mice selected for high and low antibody response to erythrocyte antigens revealed at least ten independently segregating genes affecting the antibody response (Biozzi *et al*., 1979). One of those ten loci was linked to the mouse major histocompatibility complex (MHC), H-2.

The *B* complex, the chicken MHC, plays a major role in immune responses. This group of genes also has a significant role in resistance to viral, bacterial and parasitic diseases (Taylor, 2004). The chicken MHC has *B-F*, *B-L* and *B-G* genes, which encode unique surface antigens (Pink *et al*., 1977; Nordskog *et al*., 1987; Kaufman *et al*., 1999) corresponding to class I, class II and class IV molecules, respectively. Compact *B-F/B-L* genes limit genetic recombination between these two classes (Skjodt *et al*., 1985). In contrast, there is sufficient genetic distance between *B-F/B-L* genes and B-G genes to allow recombination (Guillemot *et al*., 1989; Skjodt *et al*., 1985) as infrequent events (Hala *et al*., 1979; Koch *et al*., 1983; Briles and Briles, 1977).

MHC genotype influences antibody responses to a variety of antigens including synthetic polypeptides (Benedict *et al*., 1975; Pevzner *et al*., 1979), *Salmonella pullorum* (Pevzner *et al*., 1975) and sheep red blood

Table 1: Major histocompatibility (*B*) complex recombinant types in University of New Hampshire congenic lines. Each recombinant haplotype arose from a separate recombinational event. Individual *B* complex recombinants were backcrossed for ten generations to inbred white Leghorn Line UCD 003 (*B*17*B*17)

Congenic Line	Recombinant Designation[a]	*B-F*	*B-L*	*B-G*	Original Haplotype source[a]
003.R1	B24R1	24	24	23	B23B24
003.R2	B2R1	2	2	23	B24R1 B2
003.R3	B2R2	2	2	23	B24R1 B2
003.R4	B2R3	2	2	23	B24R1 B2
003.R5	B21R5	21	21	19	B19 B21
003.R6	B21R6	21	21	23	B2R1 B21

[a] = Miller *et al.*, 2004. Immunogenetics 56: 261-279, 2004

cells (SRBC) (Pevzner *et al.*, 1975; Loudovaris *et al.*, 1990; Dix and Taylor, 1996). For example, Pevzner *et al.* (1975) found that *B*1*B*1 chickens had lower anti-*S. pullorum* antibody than did either *B*1*B*2 or *B*1*B*19 genotype. Loudovaris *et al.* (1990) determined that MHC haplotypes affected antibody against SRBC, a T-cell-dependent antigen (McArthur *et al.*, 1973). *B*15*B*15 chickens produced much lower primary and secondary IgG and IgM antibody titers against SRBC than did *B*13*B*13-like and *B*14*B*14-like chickens.

Other examples of MHC control of the antibody response are found in lines selected for high or low antibody response to *S. pullorum* (Pevzner *et al.*, 1981), L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine acid^{10} (GAT) (Pevzner *et al.*, 1979) and SRBC (Siegel and Gross, 1980; Martin *et al.*, 1990; van der Zjipp and Nieuwland, 1986). Increased selection pressure for antibody responses altered *B* complex allelic frequencies. After 13 generations of selection for high and low SRBC response, Martin *et al.* (1990) found that the high antibody line had a 99% frequency of haplotype B21 whereas the B13 haplotype had 98% frequency in the low antibody line. A different line selected for high SRBC antibody had an increased B21 haplotype frequency while the corresponding low antibody line had an increased B14 frequency (Pinard *et al.*, 1993).

B complex congenic lines have proven to be valuable models to study the MHC effects on immune responses (Bacon, 1987; Dietert *et al.*, 1991; White *et al.*, 1994). Placing *B* complex genes on a common inbred background through repeated backcrossing minimizes nonMHC gene effects. Dix and Taylor (1996) tested two B congenic lines having the inbred Line 6_1 background that differed only by their MHC. Line 6.15-5 (*B*5*B*5) had significantly higher total anti-SRBC antibody titer than did Line 6.6-2 (*B*2*B*2) chicks at 4 and 7 wk of age. Bacon *et al.* (1987) examined eight *B* complex congenic lines developed on the inbred Line $15I_5$ background. Lines 15.C-12 and 15.N-21 had higher anti-SRBC responses than Line 15.P-13.

In light of the known *B* complex effects on immunity as well as the particular influence on antibody responses against SRBC, the objective of the current work was to examine the antibody response to sheep erythrocyte challenge in chickens that possess different MHC recombinants. Each recombinant has *B-F/B-L* genes of one MHC haplotype and the B-G genes of a distinctly different MHC haplotype. Six congenic lines, each having a unique *B* complex recombinant on the same inbred background, were evaluated for their primary and secondary antibody responses against SRBC.

Materials and Methods

Stock: Six major histocompatibility (B) complex recombinants used in this study originated from separate recombinations (Table 1). A male bearing each *B* complex recombinant (R1-R6) was crossed to highly inbred white Leghorn Line UCD 003 (*B*17*B*17) females that constituted a uniform genetic background (Fig. 1). Ten backcross generations were made by crossing recombinant heterozygous males (RB17) to UCD 003 (*B*17*B*17) females. After the tenth backcross generation, heterozygotes for each recombinant were mated *inter se* to produce progeny that were homozygous for one of six different recombinants and had 99.9% of their background genome from Line UCD 003.

The B haplotypes of each offspring generation were determined using antisera specific for the B haplotypes found in the parents (Briles and Briles, 1982; LePage *et al.*, 2000). From each bird, 0.5 mL blood was collected in 68 µM sodium citrate/ 72 µM sodium chloride anticoagulant solution. Samples were shipped on ice, overnight to Northern Illinois University. Fifty µL of 2% washed erythrocyte suspension was added to 100 µL of the antisera specific for the B haplotypes of interest in 10×77 mm tubes. Tubes were incubated at room temperature for 2 h and then overnight at 3°C. Tube contents were resuspended, incubated at room temperature for 1 h and then read for agglutination.

Eggs from each line were incubated and hatched at the University of New Hampshire Poultry Research Farm. Chicks were wing banded and vaccinated for Marek's disease at the time of hatch. Vaccination against Newcastle-bronchitis was administered at 10 days of age. Chicks were housed in heated brooder batteries for six weeks. Antibiotic-free food and water were made available *ad libitum*.

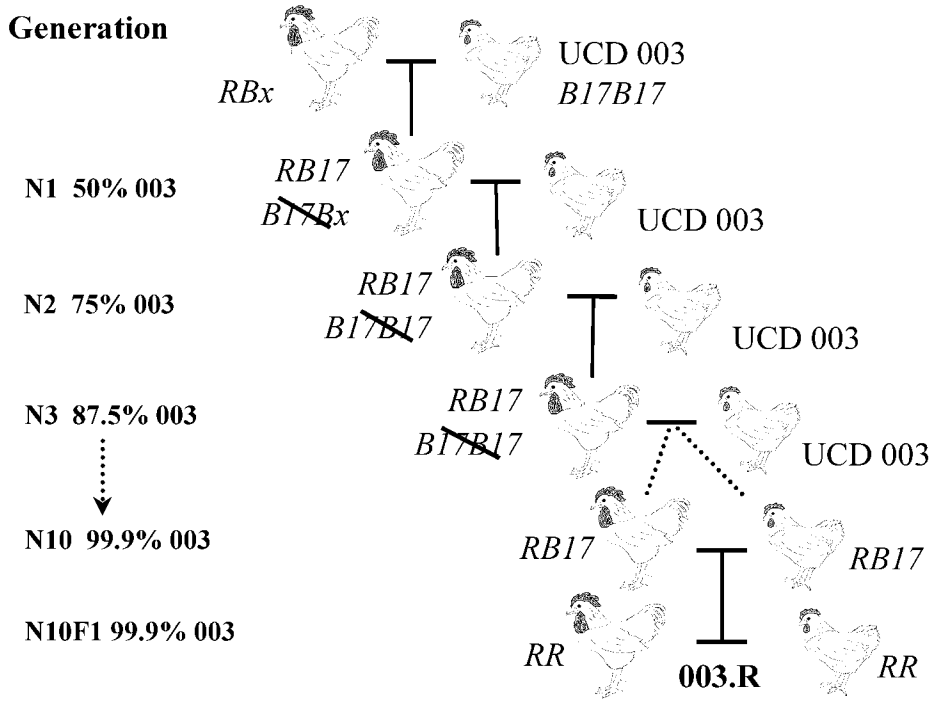

Fig. 1: Mating scheme used to produce six congenic lines containing major histocompatibility (B) complex recombinants on the inbred Line UCD 003 (*B*17*B*17) genetic background

Antigen inoculation: Sheep red blood cells were collected in Alsever's solution. Cells were washed three times and were diluted to a 2.5% vol/vol suspension in 0.9% NaCl. Three hatches totaling 117 homozygous congenic recombinant chicks from the six lines were used. The number of individual chicks per line ranged from 14 to 21. A blood sample was drawn from each chick prior to injection (D 0) to test for any cross-reacting antibodies against SRBC. Chicks were intravenously injected with 1 mL of the 2.5% washed SRBC suspension. Blood was collected 7 days post-challenge. Serum was recovered from clotted blood by centrifugation and was stored at-20°C until tested. At 11 weeks all chicks were rechallenged with 2.5% SRBC for secondary immune response.

Antibody titration: Serum complement proteins were heat inactivated by incubation at 56°C for 1 hr. Total primary and secondary anti-SRBC antibody for each bird were measured using the microtiter procedure of Wegman and Smithies (1966). The mercaptoethanol (ME)-resistant antibody against SRBC in the primary and secondary response was assayed according to the procedure of Yamamoto and Glick (1982). Titers were expressed as $\log_2$ of the reciprocal of the highest dilution exhibiting visible agglutination.

Statistical analysis: Antibody titers for primary and secondary, total and ME-resistant antibody response were analyzed by least squares ANOVA with hatch and recombinant haplotype as the main effects. Fisher's protected LSD was used to separate significant means.

Results

Four week old chicks were challenged with 1 mL 2.5% SRBC for both primary and secondary immune response. Total primary antibody response (IgM) was evaluated for homozygous *B* complex genotypes from each recombinant group (Fig. 2). Some variation was seen between R1R1, R2R2, R3R3 and R4R4 lines, which all had titers less than 5. Although R2R2 and R3R3 exhibited lower antibody levels than R1R1 and R4R4 groups, these differences were not statistically significant. R5R5 and R6R6 groups produced significantly higher antibody than did R1R1, R2R2, R3R3 and R4R4 lines. While their titers were higher than the other recombinant groups tested, R5R5 and R6R6 did not differ statistically from one another in total primary antibody production.

All challenged birds were evaluated for primary mercaptoethanol (ME)-resistant antibody production (IgG) [Fig. 3]. As IgG is a component of the total primary antibody response, these values were lower than those seen in Fig. 2. ME-resistant titers for all recombinant groups were less than 4. R3R3 had the lowest titer of all recombinant groups, while R6R6 and R5R5 had the highest titers. In spite of the variation seen between the

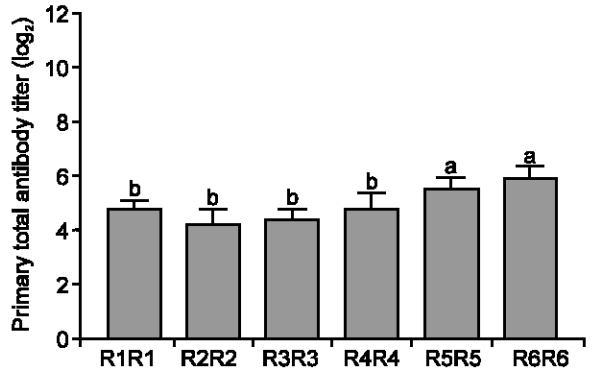

Fig. 2: Total primary anti-SRBC response for six congenic *B* complex recombinant lines to initial challenge with 2.5% SRBC suspension at 4 wk of age. Antibody titers are expressed as the log_2 of the reciprocal of the highest dilution with visible agglutination. Bars having no letter in common are considered significantly different ($p<0.05$)

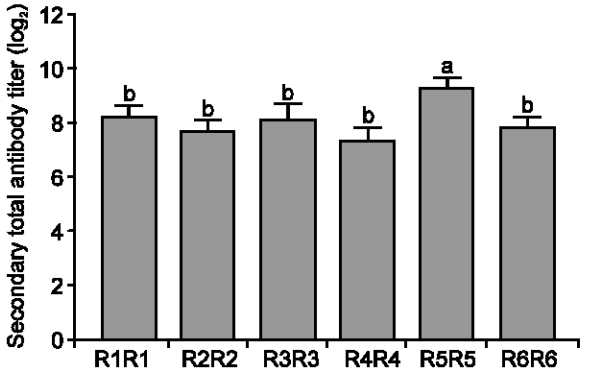

Fig. 4: Total secondary anti-SRBC response for six congenic *B* complex recombinant lines to initial challenge with 2.5% SRBC suspension at 11 wk of age. Antibody titers are expressed as the log_2 of the reciprocal of the highest dilution with visible agglutination. Bars having no letter in common are considered significantly different ($p< 0.05$)

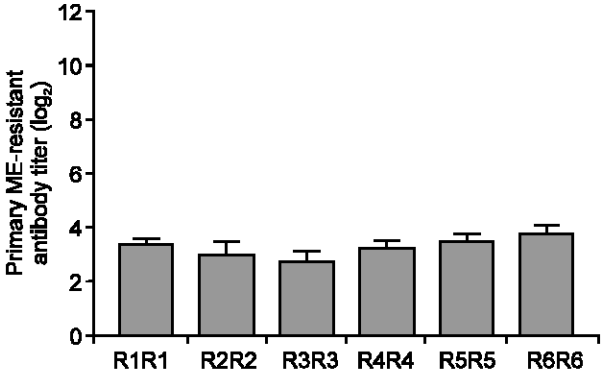

Fig. 3: Mercaptoethanol-resistant (MER) primary anti-SRBC response for six congenic *B* complex recombinant lines to initial challenge with 2.5% SRBC suspension at 4 wk of age. Antibody titers are expressed as the log_2 of the reciprocal of the highest dilution with visible agglutination. Bars having no letter in common are considered significantly different ($p<0.05$)

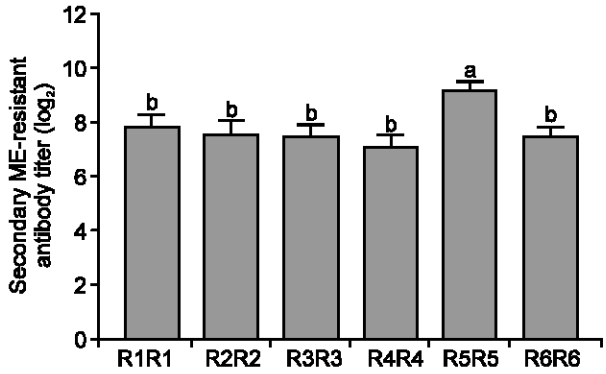

Fig. 5: Mercaptoethanol-resistant (MER) secondary anti-SRBC response for six congenic *B* complex recombinant lines to initial challenge with 2.5% SRBC suspension at 4 wk of age. Antibody titers are expressed as the log_2 of the reciprocal of the highest dilution with visible agglutination. Bars having no letter in common are considered significantly different ($p<0.05$)

various recombinant groups, the differences seen in primary ME-resistant titers did not attain statistical significance.

At 11 weeks of age, chicks were challenged with 1 mL 2.5% SRBC suspension for secondary immune response. Samples were taken one-week post inoculation and evaluated for total and ME-resistant antibody production. Total secondary antibody titers (log_2) of the different recombinant groups ranged between 7 and 9 (Fig. 4). Generally, higher amounts of antibody are produced during the secondary immune response than are seen in the primary response. R5R5 birds produced significantly higher levels of antibody than all other recombinant groups. R2R2 and R4R4 genotypes were numerically lower than R1R1, R3R3 and R6R6 but none differed statistically (Fig. 4).

Secondary antibody response is comprised mostly of IgG, the ME-resistant antibody. This fact was reflected in the results for ME-resistant secondary immune response, in that these values were only slightly lower than the total secondary antibody response (Fig. 5). There was less variation between R1R1, R2R2, R3R3, R4R4 and R6R6 lines seen in ME-resistant secondary antibody response than total secondary response.

R1R1, R2R2, R3R3 and R6R6 groups all had titers that were slightly less than 8 (log_2), while R4R4 was somewhat lower. R5R5 chickens exhibited significantly higher ME-resistant secondary antibody response than the five other congenic recombinant lines.

Discussion

This study examined the immune response to SRBC of six different (R1R1, R2R2, R3R3, R4R4, R5R5 and R6R6) congenic MHC recombinant lines of chickens. Immune response was measured as anti-SRBC antibodies that were produced during primary and secondary exposure to this antigen. Total primary antibody response was found to be higher in R5R5 and R6R6 lines in comparison to all other recombinant groups. There was no statistically significant difference between the recombinant lines for ME-resistant (IgG) primary antibody response. Secondary total and ME-resistant antibody titers were significantly higher in R5R5 chickens compared with all other groups including R6R6 birds.

Congenic lines 003.R have >99% background gene uniformity after undergoing 10 backcross generations to inbred Line UCD 003 indicating that the observed effects are attributable to MHC genes. Background gene effects other than those of the *B* complex, are minimized by the congenic mating system. Previous studies using congenic lines found that congenic 6.15-5 (*B5B5*) chickens had significantly higher antibody titers to SRBC and *Brucella abortus* (BA) compared with 6.6-2 (*B2B2*) chickens (Dix and Taylor, 1996). On the other hand, Bacon *et al.* (1987) 15.6-2 (*B2B2*) and 15.15-5 (*B5B5*) congenic lines developed on the Line $15I_5$ genetic background did not differ significantly in SRBC or BA antibody titers. These dissimilar results for the same B genotypes appear to have been influenced by the different sets of background genes. First, the two congenic series have entirely different genetic backgrounds ($15I_5$ vs. 6_1). Second, the 15.B lines tested had completed only five rather than ten backcross generations suggesting that other genes may have been present to affect the responses (Bacon *et al.*, 1987).

The higher total primary antibody response of the R5R5 and R6R6 are due to the presence of the *B-F/B-L*21 haplotype, which is associated with high antibody titers to SRBC. Previous research showed that chickens selected for high SRBC antibody titer had a B21 haplotype frequency of 80% and 99% after 10 and 13 selection generations, respectively. The low SRBC antibody line had B13 haplotype frequencies of 99% and 98% over the same selection intervals (Dunnington *et al.*, 1984; Martin *et al.*, 1990). Pinard *et al.* (1993) selected different lines for antibody response to SRBC following intramuscular injection. Haplotype frequencies increased for B21 in the high antibody line and for B14 in the low antibody line. In addition, higher antibody titers against SRBC were found in 15.B congenic lines 15.C-12 (B12B12) and 15.N-21 (*B*21*B*21) compared with Line 15.P-13 (*B*13*B*13) (Bacon *et al.*, 1987). The high and low antibody responses of haplotypes B21 and B13, respectively, corresponded to the haplotype frequency changes found by Martin *et al.* (1990).

Secondary antibody responses were significantly higher only in R5R5 chickens. The differences between the R5R5 and R6R6 secondary antibody responses are noteworthy because each line possesses the *B-F/B-L*21 haplotype with different a B-G haplotype originating from a separate recombinational event. The R5 recombinant line has the B-G19 haplotype, while the R6 recombinant line is B-G23. Neither the B-G19 nor B-G23 haplotype has been associated with high or low antibody response to challenge with sheep erythrocytes although B19 demonstrated a high response to the synthetic antigen GAT (Pevzner *et al.*, 1978). Also, the B-G region has not previously been shown to be related to superior or inferior antibody response.

Primary IgG response measured by ME-resistant antibody was not significantly different among any of the recombinant lines. This indicates that while R5R5 and R6R6 lines were more proficient at producing IgM, neither genotype was more effective at isotype switching to the more specific IgG than the other recombinant lines. Significantly higher secondary ME-resistant antibody levels were found in chickens of the R5R5 genotype. The fact that the secondary, but not the primary, IgG response differed significantly between R5R5 and R6R6 provided more evidence for genetic differences due to recombination.

The recombination events may have included a *B-F/B-L*21 gene(s) in R5 that was excluded in R6. Alternatively, a gene(s) included in the R5 B-G19 or the R6 B-G23 region may enhance or reduce the secondary response. These proposed gene disparities could impact IgG production levels thereby affecting the proportion of each antibody response that is IgG. The possibility of a genetic difference in class switching may be unlikely since this process is involved in both the primary and secondary immune responses where no consistent difference between the R5 and the R6 recombinant lines occurred. The usually dominant nature of high antibody responses over low antibody responses might increase the chance that the genetic difference enhanced rather than inhibited the antibody response. The enhancement could occur through superior macrophage antigen processing or increased SRBC-reactive T or B cells.

Antigen persistence on macrophage surfaces, attributed to slower antigen degradation, was greater in mice selected for high SRBC response (Biozzi *et al.*, 1984). Qureshi and Taylor (1993) determined that congenic 6.15-5 (*B5B5*) macrophages chickens had significantly higher SRBC phagocytosis than did 6.6-2 (*B2B2*) macrophages. These results were consistent with the

amount of SRBC antibody produced by these two lines (Dix and Taylor, 1996). Congenic lines 15.7-2 (*B2B2*) and 15.P-13 (*B13B13*) had higher SRBC phagocytic activity in macrophages elicited by Sephadex or LPS compared with lines 15.15I-5 (*B5B5*) and 15.N-21 (*B21B21*) (Puzzi *et al.*, 1990). This opposite result from the *B2B2* and *B5B5* genotypes of Dix and Taylor (1996) may be due to genetic background differences between the congenic lines. In addition, the macrophage response of congenic *B21B21* and *B13B13* chickens was contrary to the expected outcome based on the high SRBC antibody response of B21 versus B13 haplotypes. The lower SRBC phagocytosis in the *B21B21* congenics might not be common to all chickens of that genotype. Antibody responses are affected by factors in addition to phagocytic activity. For example, chickens with lower phagocytosis might have greater antigen processing efficiency. Higher antibody titers found in 003.R congenic chickens of the current study suggest that R5R5 and R6R6 macrophages may have slower, more efficient antigen processing and longer antigen persistence at the macrophage surface thus contributing to the higher response in these birds.

The data validate the utility of the congenic lines to study MHC effects on immunity. Higher primary and secondary antibody to SRBC in R5R5 and primary antibody to SRBC in R6R6 chickens point to recombination gene differences that enhance antibody production. Cellular function of T and B cells as well as macrophage antigen processing in these lines merits additional investigation. In addition, genes at the recombination point in the R5 and R6 lines should be identified. Greater knowledge of these recombinant lines will lead to a better understanding of genes affecting antibody production in chickens.

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Abbreviation Key: BA = *Brucella abortus*; Ig = immunoglobulins; MHC = major histocompatibility complex; ME = mercaptoethanol; SRBC = sheep red blood cells;