Concentration of Immunoglobulin G in Plasma Varies Among 6C.7 Recombinant Congenic Strains of Chickens

N. Yonash,1 L. D. Bacon,2 and E. J. Smith

USDA/ARS Avian Disease and Oncology Laboratory, 3606 East Mount Hope Road, East Lansing, MI 48823

ABSTRACT

Chicken Lines 63 and 72 were inbred during selection for resistance or susceptibility to viral-induced tumors. A sandwich ELISA assay was adapted to define the milligrams per milliliter of Ig-γ (IgG) in plasma from chickens of Lines 63 and 72, as well as 19 recombinant congenic strains (RCS). Each RCS resulted from a $72 \times 63$ F1 and two backcross matings using 63 as the recurrent female line. The IgG levels in the RCS were evaluated after four to seven generations of sib-matings, when each RCS was becoming inbred and fixed for a different 12.5% of the 72 genome. In three generations approximately 24-wk-old chickens of Line 72 had higher levels of plasma IgG than chickens of Line 63 ($P < 0.05$). None of the RCS had repeatable IgG levels comparable to Line 72. However, in the last two generations, two of the 18 RCS had higher IgG levels than nine with low IgG levels ($P < 0.05$). There was no correlation between an IgG level of a RCS and resistance to Marek’s disease. It was concluded that selected RCS may be useful for identifying genes that determine differences in IgG levels, as well as for understanding the relationship between genes, IgG levels, and other traits that differ between Lines 63 and 72.

(Key words: gene, inbred line, immunoglobulin, Marek’s disease, recombinant congenic strain)

INTRODUCTION

Immune competence in chickens is dependent upon cell-mediated and humoral (antibody) immune responses. In both realms, response is influenced by specific as well as non-specific factors. The genetic control of antigen-specific cell-mediated immunity (CMI) and humoral antibody response is initially impacted by the expression of MHC genes in the microenvironment of the bursa and thymus to influence the antigen response repertoire of the chicken (Veromaa and Toivanen, 1991). Subsequently, immune specificity is imparted to 1) antibody by rearrangement of Ig heavy and light chain genes in bursal cells at 10 to 15 d of embryogenesis (Masteller et al., 1997), and 2) CMI by thymic cells undergoing recombination of T-cell receptor V, D, and J genes beginning on embryonic Day 12 (Chen et al., 1996). Examples of non-antigen-specific genes that influence immunity include ones that regulate phagocytosis, e.g., cytokines, or complement proteins.

The current study was conducted to identify evidence for genes influencing another non-specific factor that may influence humoral immune response, i.e., the level of Ig-γ (IgG) proteins in plasma of peripheral blood. Rees and Nordskog (1981) described IgG differences between inbred lines of chickens that were attributable to MHC as well as non-MHC linked genes. In addition, two MHC isogenic strains have been documented in which some chickens develop dys-γ-globulinemia, i.e., absence of IgG, at adult ages (Losch et al., 1981; Erickson et al., 1982). Also, Ig levels are frequently altered if chickens are selected for various traits or, specifically, for high or low levels of IgM or IgG (Okada and Yamamoto, 1987). However, the genes regulating IgG level and the relationship of those genes to effective immune response and susceptibility to disease are not well understood. Therefore, the genetic control of chicken IgG level was evaluated in two selected inbred lines differing for tumor resistance, i.e., Lines 63 and 72 (reviewed by Bacon et al., 2000). Preliminary data indicated chickens of these lines differed for levels of IgG at 20 and 30 wk of age (L. D. Bacon and E. J. Smith, unpublished).

Recombinant congenic strains (RCS) were first conceived and developed for mice (Demant et al., 1989) as a tool to analyze complex genetic traits determined by more than one gene. The RCS are developed by a first filial generation (F1) and limited backcross matings (BCM) between two inbred strains followed by full-sib matings.

©2002 Poultry Science Association, Inc.
Received for publication December 18, 2001.
Accepted for publication March 28, 2002.
1Current Address: Department of Genetics and The Israeli Gene Bank for Agricultural Crops, The Volcani Center, P.O. Box 6, Bet Dagan, 50250, Israel.
2To whom correspondence should be addressed: baconld@msu.edu.

Abbreviation Key: BCM = backcross matings; CMI = cell-mediated immunity; F1 = first filial generation; FSM = full-sib mating; HRP = horseradish peroxidase; MD = Marek’s disease; OD = optical density; RCS = recombinant congenic strain.
(FSM) for about 20 generations. In the end, 15 to 20 RCS are developed, and given two BCM, each RCS will possess a unique random 12.5% of the donor genome in the genetic background of the recurrent parent. This constant genetic background allows the transformation of a multigenic trait into a series of single gene traits that can be easily mapped if the gene for the donor yields a measurable phenotype. In 1991, inbred Lines 63 and 72 were used to initiate the development of 6C.7 RCS of chickens to resolve genes influencing tumor resistance and immune response traits (Bacon et al., 2000). Lines 63 and 72 have the same B*2 MHC haplotype, and the resulting RCS will therefore differentiate non-MHC genes influencing trait(s) that differ in the two inbred lines. The RCS development was initiated using Line 63 as the recurrent parent. In the present study a sandwich ELISA technique was adapted to define the IgG levels in plasma from young adult chickens of inbred Lines 63 and 72, and in each of 19 6C.7 RCS that had four to seven generations of FSM. In three generations, Line 72 had higher IgG levels than Line 63 ($P < 0.05$). Moreover, an array of IgG levels was defined for the 19 RCS, and some RCS had IgG differences ($P < 0.05$) that were repeatable in succeeding generations.

**MATERIALS AND METHODS**

**Chickens**

The development of inbred Lines 63 and 72 and their various immune, virus and disease resistance traits has been reviewed (Bacon et al., 2000). The review also describes the development of the RCS using Lines 63 and 72. After a $72 \times 63$ F1 mating, Line 63 was the recurrent parent for two BCM, and then FSM were initiated in 24 families to develop RCS. The nomenclature for the 6C.7 RCS is as follows. The 6 identifies the recurrent parental line, and a C indicates this is a congenic strain. A period is inserted before identification of the line (7) from which the FSM strain. Each chicken receives a wing band with a prefix alphabetical letter that indicates the generation year at the Avian Disease and Oncology Laboratory (ADOL). The adult chickens in 1998 to 2001 were of generations G, H, I, and J.

**Immunoglobulins and Antibodies**

Affinity purified goat anti-chicken IgG (Fc fragment specific) was used as the coating antibody. The second antibody conjugated with horseradish peroxidase (HRP) was goat anti-chicken IgG. Purified reference IgG standards, substrate, and stop buffer were also supplied in a kit. The test chicken plasma was obtained by centrifugation of 3 mL of heparinized blood and was frozen at −70 C until analyzed. Analysis was done without IgG purification from the plasma.

**Sandwich ELISA**

The ELISA was modified based on the kit manufacturer’s recommendations and was conducted at room temperature. Briefly, each of the 96 wells in an ELISA plate was coated with 100 µL of a 1:100 dilution of goat anti-chicken IgG antibody for 2 h. After washing, duplicate wells received 100 µL of five dilutions of the control IgG standard: 1:30,000 = 200 ng, 1:45,000 = 135 ng, 1:60,000 = 100 ng, 1:75,000 = 80 ng, 1:90,000 = 66 ng, and 1:180,000 = 33 ng. Two wells received diluent only, and two received HRP conjugate only. The remaining 80 wells per plate were used to test 40 chickens using two appropriate dilutions of each plasma from two or three chickens of each 6C.7 RCS and Lines 63 and 72. Based on preliminary tests the plasma dilutions were 1:30,000 and 1:45,000 for Line 63, 1:60,000 and 1:90,000 for Line 72, and 1:30,000 and 1:60,000 for all 6C.7 chickens. After 2 h, the wells were washed and 100 µL HRP-labeled goat anti-chicken IgG (diluted 1:2000) was added for 30 min. After the wash, 100 µL of TMB substrate was added for 10 min. Stop buffer was added, and the blue color in the wells of the plate was analyzed on the ELISA reader at a wave length of 630 nm. One of the two dilutions of test plasma generally resulted in an optical density (OD) reading between 0.3 and 1.0, and the reading closest to 0.6 was used for analysis. If the plasma had OD outside this range, the sample was diluted at a higher or lower dilution that resulted in an OD between 0.3 and 1.0. Based on the OD readings from the IgG controls on each plate, the ELISA reader was programmed to convert the OD of the test plasma to milligrams per milliliter of IgG in the plasma.

**Statistical Analyses**

Differences in IgG levels (mg/mL) between the recombinant congenic strains, Line 63 and Line 72 within each generation (G, H, I, and J), were analyzed by one-way ANOVA using the contrast test. The ANOVA was conducted using the fit model procedure with JMP statistical package (SAS Institute, 1995). A significance level of $P < 0.05$ was used to define differences between lines.

**RESULTS**

The concentrations (mg/mL) of IgG in plasma from young adult chickens approximately 24 wk of age of Lines 63, 72, and 19 6C.7 RCS from four consecutive generations (G, H, I, and J) are recorded in Table 1. Line 72 had higher levels of IgG than Line 63 in the generations tested, i.e., G, H, and J ($P < 0.05$). Line 72 also had higher levels of IgG than any RCS with the exception of RCS 6C.7F in the H generation. Some 6C.7 RCS also differed significantly for IgG levels in each generation. In the last two generations, Strain 6C.7A had the highest IgG level of any RCS, and in the J generation the level of IgG was higher in

---

3Bethyl Laboratory, Inc., Montgomery, TX.
TABLE 1. Immunoglobulin G levels (mg/mL) and SD in 6-mo-old chickens from 6C.7 recombinant congenic strains (RCS), Lines 63, and 72 from Generations G, H, and I (1998 - 2001) and three backcross M, P, W x63 F1 from generation J (2001)

<table>
<thead>
<tr>
<th>Strain (n)</th>
<th>Mean (SD)</th>
<th>Strain (n)</th>
<th>Mean (SD)</th>
<th>Strain (n)</th>
<th>Mean (SD)</th>
<th>Strain (n)</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 (9)</td>
<td>6.91a</td>
<td>72 (7)</td>
<td>6.28b</td>
<td>72 (--)</td>
<td>NT</td>
<td>72 (10)</td>
<td>12.98a</td>
</tr>
<tr>
<td>B (12)</td>
<td>4.71</td>
<td>F (10)</td>
<td>3.29</td>
<td>63 (--)</td>
<td>NT</td>
<td>A (13)</td>
<td>5.99b</td>
</tr>
<tr>
<td>C (15)</td>
<td>3.50b</td>
<td>B (11)</td>
<td>4.94c</td>
<td>63 (--)</td>
<td>NT</td>
<td>B (20)</td>
<td>5.50b</td>
</tr>
<tr>
<td>A (14)</td>
<td>3.35c</td>
<td>C (13)</td>
<td>4.62c</td>
<td>63 (--)</td>
<td>NT</td>
<td>M (11)</td>
<td>5.34bcd</td>
</tr>
<tr>
<td>G (14)</td>
<td>3.34c</td>
<td>D (15)</td>
<td>4.54cd</td>
<td>72 (13)</td>
<td>6.91a (3.29)</td>
<td>G (23)</td>
<td>4.61cd</td>
</tr>
<tr>
<td>S (13)</td>
<td>3.32c</td>
<td>M (13)</td>
<td>4.11cdef</td>
<td>72 (12)</td>
<td>6.28a (2.25)</td>
<td>63 (10)</td>
<td>4.35def</td>
</tr>
<tr>
<td>F (10)</td>
<td>3.31cde</td>
<td>G (16)</td>
<td>4.01def</td>
<td>72 (12)</td>
<td>6.28a (7.2)</td>
<td>C (25)</td>
<td>4.35def</td>
</tr>
<tr>
<td>63 (10)</td>
<td>3.00b</td>
<td>J (9)</td>
<td>3.87bdef</td>
<td>72 (12)</td>
<td>6.28a (7.2)</td>
<td>I (13)</td>
<td>4.20def</td>
</tr>
<tr>
<td>J (15)</td>
<td>3.02b</td>
<td>63 (7)</td>
<td>3.84bdef</td>
<td>72 (12)</td>
<td>6.28a (7.2)</td>
<td>N (20)</td>
<td>4.14ef</td>
</tr>
<tr>
<td>W (14)</td>
<td>3.00def</td>
<td>A (10)</td>
<td>3.65de</td>
<td>72 (12)</td>
<td>6.28a (7.2)</td>
<td>X (15)</td>
<td>4.10ef</td>
</tr>
<tr>
<td>M (9)</td>
<td>2.99cd</td>
<td>L (8)</td>
<td>3.29cd</td>
<td>72 (12)</td>
<td>6.28a (7.2)</td>
<td>W (22)</td>
<td>4.02ef</td>
</tr>
<tr>
<td>N (15)</td>
<td>2.75cd</td>
<td>S (10)</td>
<td>3.24cd</td>
<td>72 (12)</td>
<td>6.28a (7.2)</td>
<td>F (18)</td>
<td>3.86ef</td>
</tr>
<tr>
<td>P (17)</td>
<td>2.54de</td>
<td>N (14)</td>
<td>3.14d</td>
<td>72 (12)</td>
<td>6.28a (7.2)</td>
<td>J (24)</td>
<td>3.83ef</td>
</tr>
<tr>
<td>X (10)</td>
<td>2.19ef</td>
<td>W (8)</td>
<td>2.97d</td>
<td>72 (12)</td>
<td>6.28a (7.2)</td>
<td>D (23)</td>
<td>3.66ef</td>
</tr>
<tr>
<td>T (15)</td>
<td>2.15f</td>
<td>K (11)</td>
<td>2.93d</td>
<td>72 (12)</td>
<td>6.28a (7.2)</td>
<td>D (3)</td>
<td>3.53de</td>
</tr>
<tr>
<td>R (15)</td>
<td>2.08g</td>
<td>P (14)</td>
<td>2.89f</td>
<td>72 (12)</td>
<td>6.28a (7.2)</td>
<td>S (2)</td>
<td>3.42de</td>
</tr>
<tr>
<td>L (12)</td>
<td>1.99fg</td>
<td>R (8)</td>
<td>2.95f</td>
<td>72 (12)</td>
<td>6.28a (7.2)</td>
<td>R (15)</td>
<td>3.24de</td>
</tr>
<tr>
<td>V (4)</td>
<td>1.92fg</td>
<td>X (11)</td>
<td>2.84e</td>
<td>72 (12)</td>
<td>6.28a (7.2)</td>
<td>R (20)</td>
<td>3.20fg</td>
</tr>
<tr>
<td>I (13)</td>
<td>1.82g</td>
<td>T (4)</td>
<td>2.63f</td>
<td>72 (12)</td>
<td>6.28a (7.2)</td>
<td>T (17)</td>
<td>3.19fg</td>
</tr>
<tr>
<td>K (13)</td>
<td>1.80h</td>
<td>V (4)</td>
<td>2.37g</td>
<td>72 (12)</td>
<td>6.28a (7.2)</td>
<td>L (7)</td>
<td>3.08fg</td>
</tr>
</tbody>
</table>

*Mean IgG levels with no common superscript differ (P < 0.05) using test within generation.

1In generation I, only males were used and Lines 63 and 72 were not tested (NT).

2Backcross chickens were only produced and evaluated in Generation J.

Young adult chickens of inbred Line 63 had lower levels of plasma IgG than chickens of Line 72 in three generations, as measured by a sensitive ELISA test (P < 0.05). This result was predicted based on earlier limited observations (1980) between these lines using radial immunodiffusion tests (L. D. Bacon and E. J. Smith, unpublished). In 1980, the lines did not differ for IgM level at any age, and IgG levels were similar at 6 wk, but at 20 and 30 wk of age Line 72 had more IgG than Line 63 (P < 0.05). Therefore, precise unbiased evaluations were conducted using recently developed ELISA procedures to analyze IgG in plasma from young adult chickens. The authors know of no other papers describing differences in Ig levels between these lines.

As described in the introduction, 6C.7 RCS are under development to identify genes outside the MHC that influence tumor resistance, as well as genes that control immune-associated traits that differ between the base lines 63 and 72. Here the IgG levels in 19 of the 6C.7, R, P, T, V, N, X, I, and W (P < 0.05). In the last two generations (I, J), this result was also observed for Strain 6C.7A. Thus, in the last two generations (I, J) 6C.7 Strains A and B have higher IgG levels than Strains K, R, P, T, V, N, X, I, and W (P < 0.05).

In Generation J three of the 6C.7 RCS were backcrossed to Line 63, and the level of IgG in the plasma of three to five males per strain was measured. Strain 6C.7M had 5.34 mg/mL of IgG compared to 6.51 mg/mL in Mx63 backcross chickens. Strain 6C.7W had 4.02 mg/mL compared to the Wx63 backcross chickens with 5.97 mg/mL (P < 0.05). Strain P had 3.42 mg/mL compared to Px63 with 3.50 mg/mL. Thus the level of IgG in the backcross chickens was elevated compared to the 6C.7 parental strain, but the increase was only significant in Strain 6C.7W. Note that the level of IgG was higher in the Mx63 and Wx63 than Px63 backcross chickens (P < 0.05).

**DISCUSSION**

Young adult chickens of inbred Line 63 had lower levels of plasma IgG than chickens of Line 72 in three generations, as measured by a sensitive ELISA test (P < 0.05). This result was predicted based on earlier limited observations (1980) between these lines using radial immunodiffusion tests (L. D. Bacon and E. J. Smith, unpublished). In 1980, the lines did not differ for IgM level at any age, and IgG levels were similar at 6 wk, but at 20 and 30 wk of age Line 72 had more IgG than Line 63 (P < 0.05). Therefore, precise unbiased evaluations were conducted using recently developed ELISA procedures to analyze IgG in plasma from young adult chickens. The authors know of no other papers describing differences in Ig levels between these lines.

As described in the introduction, 6C.7 RCS are under development to identify genes outside the MHC that influence tumor resistance, as well as genes that control immune-associated traits that differ between the base lines 63 and 72. Here the IgG levels in 19 of the 6C.7 RCS have been characterized. In the last two generations, Strains A and B had higher IgG levels than strains K, R, P, T, V, N, X, I, and W (P < 0.05). Occasionally a 6C.7 strain even had a lower level of IgG level than 63 (P < 0.05); i.e., 6C.7K and I in the G and 6C.7V in the I generation. Thus the random assortment of genes from Line 72 may also result in an assortment of genes that reduces the level of IgG below that of the low line, i.e. 63. Now that 6C.7 RCS have been identified that differ in the level of IgG at adulthood, it would be desirable to study IgG at other ages in high vs. low RCS, and to also evaluate those lines for IgM and possibly IgA differences.

Four of the 6C.7 strains have been backcrossed to Line 63 to obtain adequate breeder chickens for continued reproduction and development, i.e., 6C.7T in the H generation, 6C.7F in the I generation, and 6C.7L and V in the J generation.
resource population of several hundred F2 chickens that
to handle selection for low group productivity and survivability (Cheng et al., 2001). Hence, it may be of interest that strains 6C.7T, L, and V had the lowest levels of IgG among the various 6C.7 strains when these reproduction crises occurred, whereas strain 6C.7F had a high IgG level. Thus, there may be some association between extreme IgG levels and reproductive performance, i.e., common genes may influence these traits. Recently a high level of γ-globulin has been associated with low fertility (Chao and Lee, 2001) and with selection for low group productivity and survivability (Cheng et al., 2001).

The 6C.7 RCS may be useful for the identification of genomic regions or actual genes controlling IgG levels. This process could involve the creation of a segregating resource population of several hundred F2 chickens that are progeny of F1 parents derived from matings between 6C.7 strains with high or low IgG levels, e.g., strains 6C.7B and 6C.7R. Characterization of the F2 population for IgG level by selective genotyping of the extreme chickens (10 to 15% highest and lowest IgG levels) using DNA markers may lead to identification of QTL associated with IgG level. This resource population could also be used to identify candidate genes previously associated with Ig levels in the human or mouse. If markers are identified, then their association with resistance to various diseases can be monitored.

Lines 63 and 72 differ for IgG heavy chain allotype loci, i.e., Line 63 has the G1*E, and 72 has G1*A (Benedict, 1979). However, we do not believe allotype antigenic differences were responsible for the differences in the IgG levels. Allotypic markers are definable within a species. However, when a species (goat) is immunized with Ig of another species (chicken) they are not known to produce antibodies to the Ig allotypes. To reduce this possibility the goat anti-chicken IgG in the kit was produced using serum IgG from several hundred chickens of several breeds (personal communication, Bethyl Laboratory, 2001). Furthermore, the data for Lines 63 and 72 were parallel with the IgG control standard for the dilutions tested, indicating the goat anti-chicken IgG was reacting equivalently to IgG from both strains. We do not know of an association between Ig allotypes and IgG levels in plasma in any species. The IgG allotype genes have been analyzed for affects on tumor resistance and had no influence on herpesvirus-induced Marek’s disease (MD) or regression of Rous sarcoma tumors. However, they were associated with a recessive resistance to B-cell lymphoma genesis (lymphoid leukemia) in F3 chickens of lines 63 and 63 (Bacon et al., 1986). Line 100 is highly congenic to Line 72 (see Bacon et al., 2000), and in 1986 Line 100 and Line 63 were both susceptible to a Subgroup A avian leukemia virus that was used to induce sarcomas and lymphoid leukemia. It is of interest that Lines 63 and 72 also differ dramatically in the size of their primary lymphoid organs, i.e., the bursa of Fabricius and the lobes of the thymus are smaller in Line 63 (Lee et al., 1981; Powell et al., 1982). Furthermore, lymphoproliferation traits are higher in Line 72 than in Line 63, i.e., graft vs. host response in vivo (Pazderka et al., 1975), in vitro response of lymphocytes to mitogens (Lee and Bacon, 1983), or mixed lymphocyte cultures (Bacon and Lee, 1981). Data also indicate that Lines 63 and 72 differ in production of interferon in the supernatants of mitogen stimulated white blood cells (Bacon, unpublised data). No direct relationship between these traits and IgG levels is known. However, it is of interest that γ-interferon is the main factor controlling Ig class switching in mice (see Boehm et al., 1997).

Specific antibodies in immunoglobulins are generally associated with immune resistance to extracellular bacteria and viruses. Therefore IgG levels may be most relevant to diseases caused by extracellular microbes (Sarker et al., 2000; Li et al., 2000) or to autoimmune diseases that are frequently associated with antibodies (Luster et al., 1977). At present, the 6C.7 strains have only been studied for resistance to MD. Lines 63 and 72 differ dramatically for MD resistance as well as IgG levels, and therefore it is plausible IgG levels may be related to resistance. Moreover, Okada and Yamamoto (1987) reported that a chicken line selected for decreased IgG was more susceptible to MD than a line selected for decreased IgG. Initial analyses of the 6C.7 strains has indicated that Strains 6C.7M, P, and W are relatively susceptible to MD, whereas the other strains have MD resistance like Line 63 (Bacon et al, 1996; Yonash et al., 1998). The 6C.7M strain had a higher level of IgG than the 6C.7P strain in Generations H, I, and J, and the 6C.7W strain had intermediate levels of IgG in all generations. Thus, there is no consistent correlation between the level of IgG and MD resistance in the 6C.7 strains. This result might have been anticipated, as studies indicate CMI is more important than antibody response in resistance to MD (see Calnek and Witter, 1997).

ACKNOWLEDGMENTS

The authors thank Chen Lo Chen and Henry Hunt for constructive editorial comments and Evelyn Young and Cecyl Fisher for excellent technical assistance.

REFERENCES


