ABSTRACT  To determine the role of genetics in baseline lymphocyte parameters, several distinct lines of chickens were examined for differences in peripheral blood leukocyte (PBL) populations. Four highly inbred chicken lines (MHC congenic Fayoumi lines M15.2 and M5.1, and MHC congenic Leghorn lines G-B1 and G-B2), two advanced intercrosses [F5 (Broiler × G-B2) and F5 (Broiler × M15.2)], and an outbred population of broilers were used. Leukocytes isolated from healthy adult birds were labeled with monoclonal antibodies: chCD3, chCD4, chCD8, chBu-1, and hCD14. Flow cytometry was used to determine the total percentage of positively labeled cells for each surface marker in a sample, as well as the mean fluorescent intensity, or surface marker density, of a labeled subset. Significant line differences for percentage positive CD3 T cells and the ratio of B cells:T cells (represented by the Bu-1:CD3 ratio) were found. The effect of line was also significant for CD3 and CD8 T cell receptor density. Effects of sex and MHC on PBL cell surface marker expression were not significant in the lines examined. This study demonstrates the effect of genetic line on resting leukocyte composition of peripheral blood in the chicken lines examined. Observed PBL differences add to our growing knowledge of the varied roles that immune system status (defined by specific cell populations) and genetic background have in determining susceptibility and disease progression in chickens.

(Key words: B cell, genetic difference, peripheral blood leukocyte, major histocompatibility complex congenic, T cell)

INTRODUCTION

There are many ways to distinguish the several leukocyte populations from each other. Location, activity assays, size, and surface markers are some of the most common means. Surface markers are probably the most used because of the wide availability of specific antibody reagents (Chung et al., 1991). Lymphocytes arise from progenitor stem cells that, through the complex process of differentiation, produce each of the many cell types of the immune system. T cells generally express the CD3 complex early in cell development and expression remains throughout the life of each individual T cell (Chan et al., 1988; Janeway et al., 2001). T cells can be further classified by cell development and expression of the coreceptor molecules CD4 and CD8. Cytotoxic T cells typically express CD8 compared with helper T cells that express CD4 (Chan et al., 1988; Janeway et al., 2001). CD14 and TLR4 molecules are expressed on the surface of many phagocytic cell types such as monocytes, macrophages, neutrophils, and dendritic cells (Janeway et al., 2001). B cells can be distinguished by the expression of IgM or other surface molecules such as Bu-1 (Veromaa et al., 1988; Janeway et al., 2001).

Significant peripheral blood leukocyte (PBL) composition differences exist in the MHC congenic chicken lines designated CB and CC. Hala et al. (1991) found a higher percentage of CD4+ PBL in the CB line compared with a higher number of CD8+ PBL in the CC line, and concluded that MHC played an important role in determining T cell PBL composition. These 2 lines were later shown to have markedly different CD4, CD8, and CD4:CD8 ratio populations in peripheral blood samples obtained at 7-d intervals over a 6-wk posthatch period (Hala et al., 1992).

Another study that examined PBL populations of 2 inbred chicken lines using flow cytometry found differences in CD4, CD8, and B cell numbers, but not CD4:CD8 ratios (Burgess and Davidson, 1999). Line 72 had higher numbers of CD4 and CD8 T cells and B cells compared with line 61 in peripheral blood samples, but significant differences could not be demonstrated for CD4:CD8 ra-

Abbreviation Key: FITC = fluorescein isothiocyanate; LS mean = least squares mean; PBL = peripheral blood leukocyte.
Birds meeting or exceeding NRC (1994) requirements. Individually caged with ad libitum access to water and feed were used. All birds appeared healthy and were indi-

tively sampled. Similar numbers of males and females per broilers, 6 G-B1, 5 G-B2, 5 M5.1, and 4 M15.2 birds were from wing or jugular veins in 10-mL syringes preloaded from adult chickens 38 to 40 wk in age were collected of the 6 collection dates.

To minimize the potential effect of intercross lines, F5 (Br × G-B2) and F5 (Br × M15.2). Nine outbred broiler breeder males (Kaiser et al., 1998) were mated to G-B2 and M15.2 females and the resulting progeny were intermated (within dam line) for 4 generations to produce the advanced intercross lines. Ten birds were sampled from each of the 2 advanced intercross lines, F5 (Br × G-B2) and F5 (Br × M15.2). Nine broilers, 6 G-B1, 5 G-B2, 5 M5.1, and 4 M15.2 birds were also sampled. Similar numbers of males and females per line were used. All birds appeared healthy and were individually caged with ad libitum access to water and feed meeting or exceeding NRC (1994) requirements.

MATERIALS AND METHODS

Birds

The G-B1 and G-B2 are MHC congenic lines of Leghorn chickens that originated in 1965, and the MHC congenic lines M15.2 and M5.1 are Egyptian Fayoumi and were established in 1954 (Zhou and Lamont, 1999). The G-B1 and G-B2 Leghorn congenic pair and the M15.2 and M5.1 Egyptian Fayoumi congenic pair each share an identical genetic background and differ only in the microchromosome bearing the MHC. These 4 lines are highly inbred, each with an inbreeding coefficient of 0.99 (Zhou and Lamont, 1999). Outbred broiler breeder males (Kaiser et al., 1998) were mated to G-B2 and M15.2 females and the resulting progeny were intermated (within dam line) for 4 generations to produce the advanced intercross lines. Ten birds were sampled from each of the 2 advanced intercross lines, F5 (Br × G-B2) and F5 (Br × M15.2). Nine broilers, 6 G-B1, 5 G-B2, 5 M5.1, and 4 M15.2 birds were also sampled. Similar numbers of males and females per line were used. All birds appeared healthy and were individually caged with ad libitum access to water and feed collection, preparation, and cell surface labeling

Individual birds were assayed on 1 of 6 collection dates over a period of 8 d. To minimize the potential effect of assay date on study results, each line was tested on each of the 6 collection dates.

Peripheral blood samples (approximately 5 to 10 mL) from adult chickens 38 to 40 wk in age were collected from wing or jugular veins in 10-mL syringes preloaded with 0.5 mL of sterile 0.5 M EDTA to prevent clotting. Leukocytes were separated from red blood cells using Histopaque 10773 by centrifugation for 30 min at 400 × g. The leukocytes, contained in theuffy coat, were initially washed 3 times in PBS by centrifugation for 10 min at 400 × g, pelleted, and resuspended in PBS. Leukocyte cell counts and viability were determined by using Trypan Blue exclusion.

Isolated chicken leukocytes were incubated with the following monoclonal antibodies: chCD3 fluorescein isothiocyanate (FITC), chBu-1 (FITC), chCD4 (FITC), chCD8 phycoerythrin, and hCD14 (FITC). All antibodies are species-specific with the exception of CD14, which has been shown to label chicken CD14 cells (Dil and Qureshi, 2002). A total of 1 × 10^6 cells from each chicken were incubated for 1 h at room temperature with antibody concentrations as follows: chCD3 (FITC) 4.0 µg/10^6 cells, chBu-1 (FITC) 2.0 µg/10^6 cells, chCD4 (FITC) 1.0 µg/10^6 cells, chCD8 (phycoerythrin) 1.0 µg/10^6 cells, and hCD14 (FITC) 1:4 volume ratio. Leukocyte samples were dual labeled with CD4 and CD8 antibodies in a 2-step manner, and all other samples were singularly labeled with the remaining antibodies. Following antibody incubation, all samples were washed 3 times in PBS by centrifugation for 10 min at 400 × g, pelleted, and resuspended in PBS with 0.1% sodium azide. Following the final wash cycle, samples were resuspended in 0.5 mL of PBS with 1% formaldehyde and held at 4°C overnight before Fluorescence Activated Cell Sorter5 processing. Fluorescence activated cell sorting is a method used to count and sort cells based on size and fluorescence. Flow cytometric analysis of all samples was performed at the Iowa State University Cell and Hybridoma Facility. Samples were analyzed for percentage of total population positive for the cell surface marker examined and for fluorescent intensity. Fluorescent intensity is the relative degree of antibody binding to the cell surface on leukocytes that are positive for the specific marker.

Statistical Analysis

All data were analyzed using JMP software (SAS Institute, 2000) to determine least squares mean (LS means) and P values for parameters tested. The GLM procedure of SAS (SAS Institute, 2000) was used for the following model:

\[ y = u + \text{line} + \text{sex} + \text{assay date} + e \]

Line and sex were considered fixed effects, and assay date was a random effect. Statistically significant differences between individual line data (LS means) were determined using Student's t-test (P < 0.05). Two-way interactions, line × sex, sex × assay date, and line × assay date, were initially tested and found insignificant (P > 0.1). These two-way interactions were excluded from the final model.
in this study, it does agree with the overall findings of leukocyte percentages among the chicken lines examined. Current observation of a significant line effect for CD3 types. Although the Smyth line study conflicts with our Smyth (1996) found no differences in PBL composition served in this study with the exception of CD3. Erf and lymphoid organs such as the spleen or lymph nodes. CD3 T cell population might be found residing in specific these congenic lines, a higher percentage of the whole T cell populations were observed to be 2-fold lower in the current study's experimental design was the sampling of each line on the 6 assay dates to minimize confounding of potential assay date and genetic line effects. In comparing the LS mean percentages of leukocytes that expressed the tested cell surface receptors, there was a significant line difference \( P = 0.04 \) in the percentage of CD3 positive cells but not for the other cell surface markers examined. For the CD3 marker, G-B1 and G-B2 birds had lower percentages of observed leukocyte populations compared with the other lines examined. The MHC congenic Leghorn lines had CD3 population percentages that were 2-fold lower than the other lines (Table 1). These data suggest that the Leghorn lines have a lower percentage of peripheral CD3 positive cells. Another explanation could be that, although peripheral blood CD3 T cell populations were observed to be 2-fold lower in these congenic lines, a higher percentage of the whole CD3 T cell population might be found residing in specific lymphoid organs such as the spleen or lymph nodes.

In general, PBL composition differences were not observed in this study with the exception of CD3. Erf and Smyth (1996) found no differences in PBL composition of Smyth line chickens for CD3, CD4, CD8, and B cell types. Although the Smyth line study conflicts with our current observation of a significant line effect for CD3 leukocyte percentages among the chicken lines examined in this study, it does agree with the overall findings of the present study that indicate no differences in PBL for the other cell surface markers. Parmentier et al. (1995) found no significant PBL subset differences in percentages of CD3, CD4, and CD8 cells in 2 chicken lines divergently selected for antibody response to SRBC. However, the same study found line differences in CD4 and CD8 cell populations of spleen and thymus tissue samples. In light of these findings, it is possible that the 7 chicken lines of the current study may have measurable lymphocyte differences in spleen and thymus tissues that were not reflected in our study of PBL.

Closer examination of percentages of cells positive for the surface markers CD3, CD4, and CD8 showed an interesting phenomenon (Table 1). The percentage of CD3 positive cells is lower than the sum of CD4, CD8, and CD4+CD8+ T cell percentages (data not shown). This discrepancy is highest for the 2 Leghorn inbred lines, G-B1 and G-B2. There is no clear explanation for the observed inconsistency in CD3 cell percentages compared with those obtained when CD4, CD8, and CD4+CD8+ T cells are added, but CD4 can be expressed on monocytes and macrophages (Janeway et al., 2001). The monoclonal antibody used to stain CD3 cells may have had a lower affinity for the targeted surface marker compared with the 2 other antibodies, CD4 and CD8. The same T cell population labeling inconsistencies have been observed in other studies. Maslak and Reynolds (1995) examined lymphocyte concentrations in the Harderian gland of chicks at 1, 4, and 8 wk of age. Lymphocyte subset profiles of that study at all 3 times showed lower CD3 cell numbers, compared with the sum of CD4 and CD8 positive cells. More recently, Withanage et al. (1998), found similar lower CD3 cell numbers compared with the sum of CD4 and CD8 positive cells in the ovaries, uterus, and vagina of laying hens after experimental exposure to Salmonella enteritidis. Although Withanage et al. (1998) examined tissue samples for changes in T cell subsets and the current study used peripheral blood cells, both studies used the same antibody clones from the same vendor. Therefore, it is likely that the choice of monoclonal antibody, especially

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**RESULTS AND DISCUSSION**

**Peripheral Blood Leukocyte Percentages**

The LS mean percentages for the effects of line, sex, and assay date on tested cell surface markers are shown in Table 1. The effect of sex was not significant (Bu-1, CD3, CD14, CD4, CD8, and CD4+CD8), and is consistent with previous work by Hala et al. (1991), in their investigation of MHC congenic chickens for CD4 and CD8 PBL differences. The effect of assay date was significant for the tested cell surface markers, with the exception of CD4 and CD8 percentage positive cells. Incorporated into this study's experimental design was the sampling of each line on the 6 assay dates to minimize confounding of potential assay date and genetic line effects.

In comparing the LS mean percentages of leukocytes that expressed the tested cell surface receptors, there was a significant line difference \( P = 0.04 \) in the percentage of CD3 positive cells but not for the other cell surface markers examined. For the CD3 marker, G-B1 and G-B2 birds had lower percentages of observed leukocyte populations compared with the other lines examined. The MHC congenic Leghorn lines had CD3 population percentages that were 2-fold lower than the other lines (Table 1). These data suggest that the Leghorn lines have a lower percentage of peripheral CD3 positive cells. Another explanation could be that, although peripheral blood CD3 T cell populations were observed to be 2-fold lower in these congenic lines, a higher percentage of the whole CD3 T cell population might be found residing in specific lymphoid organs such as the spleen or lymph nodes.

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**TABLE 1. Least squares means ± SE and P-values for percentage positive leukocytes expressing cell surface antigens for Bu-1, CD14, CD3, CD4, CD8, and CD4+CD8+ double positive**

<table>
<thead>
<tr>
<th>Cell surface antigens</th>
<th>Bu-1</th>
<th>CD14</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD4+CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broiler</td>
<td>7.30±0.90</td>
<td>25.07±3.13</td>
<td>14.88±1.98&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>10.05±1.86</td>
<td>7.12±1.64</td>
<td>1.12±0.21</td>
</tr>
<tr>
<td>F&lt;sub&gt;3&lt;/sub&gt; (Br × G-B2)</td>
<td>6.79±0.86</td>
<td>21.15±2.97</td>
<td>16.32±1.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.63±1.76</td>
<td>12.96±1.57</td>
<td>1.25±0.20</td>
</tr>
<tr>
<td>F&lt;sub&gt;5&lt;/sub&gt; (Br × M5.2)</td>
<td>5.69±0.84</td>
<td>21.72±2.98</td>
<td>16.71±1.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.70±1.75</td>
<td>7.75±1.54</td>
<td>1.31±0.20</td>
</tr>
<tr>
<td>G-B1</td>
<td>6.15±1.00</td>
<td>14.99±3.75</td>
<td>9.17±2.40&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>11.84±2.26</td>
<td>11.45±1.99</td>
<td>0.96±0.26</td>
</tr>
<tr>
<td>G-B2</td>
<td>6.39±1.21</td>
<td>17.19±4.28</td>
<td>7.71±2.67&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.02±2.51</td>
<td>11.53±2.21</td>
<td>1.13±0.29</td>
</tr>
<tr>
<td>M15.2</td>
<td>7.29±1.26</td>
<td>21.75±4.64</td>
<td>12.28±2.95&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>6.70±2.77</td>
<td>7.58±2.44</td>
<td>1.12±0.32</td>
</tr>
<tr>
<td>M5.1</td>
<td>7.44±1.23</td>
<td>21.02±5.29</td>
<td>16.14±2.72&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>12.96±2.55</td>
<td>10.77±2.25</td>
<td>1.47±0.29</td>
</tr>
</tbody>
</table>

<sup>P-value</sup>
| Line                  | 0.83 | 0.58 | 0.04 | 0.17 | 0.12 | 0.75 |
| Sex                   | 0.32 | 0.96 | 0.49 | 0.91 | 0.62 | 0.08 |
| Assay date            | 0.00 | 0.00 | 0.00 | 0.62 | 0.46 | 0.00 |

<sup>a–c</sup>Means within a column that do not share a common superscript differ significantly \( P < 0.05, \) Student’s \( t \)-test.

<sup>1</sup>Leukocytes expressing both CD4 and CD8 cell surface antigens.
CD8, has an influence on labeling percentages measured for T cells.

**Lymphocyte Surface Antigen Intensity**

The effect of sex on fluorescent intensity of leukocytes positive for a specific cell surface antigen was not significant (Table 2). The effect of line on fluorescent intensity of surface marker expression for the Bu-1 and CD4+CD8+ double positive T cells approached significance with P values of 0.06 and 0.09, respectively.

Genetic line had a significant effect on LS mean intensity of leukocytes expressing the surface markers CD8 and CD3 (Table 2). Intensity for CD8, which corresponds to the cytotoxic T lymphocyte population in the peripheral blood sample, was highly significant. The M5.1 line had the highest level of CD8 co-receptor fluorescent intensity, which was twice the measured level for the G-B1, G-B2, and F5 (Br X G-B2) lines (Table 2). The broiler, M15.2, and F5 (Br X M15.2) lines had similar levels of CD8 fluorescent intensity values for CD8. The G-B1 and F5 (Br X G-B2) lines had the lowest observable LS mean fluorescent intensity for the CD8 cell surface co-receptor.

The effect of line, and therefore genetic background, on fluorescent intensity of leukocytes expressing the CD8 surface co-receptor was of the greatest statistical significance in this study (Table 2). Among the lines, there were vast differences in cytotoxic T cells with regard to their surface receptor density. The recognition of MHC-presented peptide antigens and cytolytic activity is shown to be greatly enhanced by the presence of CD8 surface proteins on T cells (Cho et al., 2001). Human CD8 is a co-receptor that intimately interacts with the MHC-peptide structure and enhances antigen sensitivity for T cell cytolytic activity (Purbhoo et al., 2001). The higher density of CD8 surface receptors in certain lines, such as the M15.2, M5.1, and the F5 (Br X M15.2) birds, could be the result of enhanced transcriptional mechanisms or of selective pressures favoring the maturation of CD8 T cells that express high levels of this surface receptor.

The effect of chicken line on CD3 cell fluorescent intensity was also significant (Table 2). The G-B2 line had the highest observed fluorescent intensity, whereas the F5 (Br X M15.2) line had the lowest measured CD3 surface antigen density of all the lines. In general, the 2 MHC congenic Leghorn lines, G-B1 and G-B2, had similar levels of CD3 intensity, which were approximately 1.5-fold greater than that of all the other lines.

The effect of genetic line was significant on both percentage positive and fluorescent intensity of the CD3 surface receptor (Tables 1 and 2). This suggests that genetic line has a strong effect on CD3 positive T cells, not only on percentage in peripheral blood cells, but in surface receptor density on these cells as well. The CD3 surface protein is intimately associated with the T cell receptor, which recognizes MHC class I and II presented peptides (Janeway et al., 2001). It would be of interest to determine if T cell receptor density expression is also controlled by genetic line. Use of another T cell surface marker such as CD45 would provide additional information about total T cell population measurements in future studies.

In studies of the immune system, inbred strains are often used to limit the potential effects of genetic variation (Yamakawa et al., 1996; Knippeles et al., 1999). Two mouse strains with immune system response differences are BALB/c and C57BL/6. BALB/c mice have more dominant humoral immune responses, whereas in C57BL/6 mice, the cell-mediated immune response is dominant (Karp et al., 1994). The C57BL/6 mice have lower percentages of thymic CD4 T cells than BALB/c mice (Duarte and Penha-Goncalves, 2001).

The 2 sets of MHC congenic pairs had similar within-pair CD3 surface receptor densities (Table 2), in agreement with the general findings of the present study that MHC had little influence on the composition of PBL of adult chickens. The non-MHC genes involved in leukocyte composition are likely to be multifactorial in nature and remain to be fully elucidated.
Cell Population Ratios

The primary flow cytometric data were used to examine ratios of specific leukocyte populations that were positively labeled with the surface marker. The ratio of CD4:CD8 T cells was used to estimate the helper T cell:cytotoxic T cell ratio in chicken peripheral blood. Genetic line shows a strong influence in determining the CD4:CD8 ratio in different strains of mice (van Meerwijk et al., 1998; Duarte and Penha-Gonçalves, 2001). In the present study, however, the effect of genetic line on CD4:CD8 ratio was not significant. The ratio of CD14:CD3+Bu-1 cells, which correlates to the heterophil:B + T cell ratio, was also examined. The effect of line on the ratio of CD14:CD3+Bu-1 leukocyte cells was not significant in this study.

Line effects on the Bu-1:CD3 ratio, which estimates B cell:T cell ratios, were observed (Table 3). The G-B2 line had the highest Bu-1:CD3 ratio, whereas the F5 (Br × M15.2) line had the lowest ratio (Table 3). The 5 remaining lines had Bu-1:CD3 ratios that were not significantly different and ranged from 0.56 to 0.91. Significant differences in B cell:T cell population, as estimated by the Bu-1:CD3 ratio, suggest that further examination is warranted to determine whether these lines are predisposed to respond to a pathogen either with a humoral or cell-mediated basis, reflective of the overall peripheral blood lymphocyte composition.

The differences in the B cell:T cell ratios in this study are a result of varied levels of CD3 cells, not B cells, in peripheral blood (Table 1). Observed B cell percentages were very consistent among lines, in comparison with the more widely distributed T cell numbers for PBL (Table 1). Although Burgess and Davison (1999) found no B cell:T cell ratio differences in their chicken lines, our experiment used a CD3 receptor as an indicator of the whole T cell population in PBL samples, compared with their combination of CD4 and CD8 positive cells to estimate the same leukocyte subset.

In the present study, we observed small percentages of CD4+ CD8+ double positive T cells in all 7 chicken lines. These data agree with a study that found this novel T cell subset in peripheral blood and tissues such as the spleen and intestinal epithelium of chickens (Luhtala et al., 1997). Associations of age and dominant Mendelian inheritance patterns of double positive T cell abundance have also been reported (Luhtala et al., 1997; Erf et al., 1998). The presence of CD4+ CD8+ T cells is well established, but no clear function has yet been recognized in the chicken. Analysis of double positive T cells in the peripheral blood of humans and pigs suggests their role as memory T cells (Zuckermann, 1999).

The genetic lines studied allowed the opportunity to contrast the effect of MHC and non-MHC genetic variation on lymphocyte subsets. Based on the 2 MHC congenic pairs in this study, the results suggest that the non-MHC genes, in total, had a larger effect on the lymphocyte characteristics than did the variation in the MHC haplotypes. Of all the assays conducted, only a single contrast within a congenic pair (fluorescent intensity for CD8, Table 2) was significant. This result may be specific to the individual MHC haplotypes examined, or may be a more general phenomenon reflecting the multigenic control of cell surface protein receptor expression. Results of this flow cytometric study suggest that genetic line influences specific subsets of PBL composition in chickens.

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