TOLERANCE AND AUTOIMMUNITY TO ERYTHROID DIFFERENTIATION (B-G) MAJOR HISTOCOMPATIBILITY COMPLEX ALLOANTIGENS OF THE CHICKEN*

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On the basis of biochemical, genetic, and biological data, the chicken major histocompatibility complex (MHC) has been divided into three subregions: B-L, B-F, and B-G. Unlike the B-F and B-L regions, which code for homologues of mammalian class I and class II MHC antigens, there is no known mammalian homologue of B-G antigens. B-G antigens are erythroid differentiation antigens as they are restricted in their expression to erythrocytes (RBC) and erythroid progenitors. This raises the question whether the B-G locus should be considered as part of the MHC or whether it should be considered as just another blood group locus fortuitously linked to the chicken MHC. However, B-G genes resemble classic MHC genes in the sense that (a) the gene products are highly polymorphic and the degree of polymorphism is at least comparable to that exhibited by class I antigens of the chicken and the mouse, (b) they exist in strong linkage disequilibrium with class I (B-F) genes in outbred populations, and (c) they share several of the same immunological characteristics as classic MHC antigens as they are more immunogenic and less tolerogenic than other non-MHC antigens. Because B-G antigens are expressed only on erythrocytes and their progenitors, it is no surprise that they are not involved as targets for skin graft rejection or graft-vs.-host (GVH) reactions. However, these antigens might be expected to serve as effective barriers or targets for rejection of erythroid chimerism. These experiments were partially designed to answer this question.

A second interesting property of B-G antigens is that all animal species that have been studied, including alligators, chickens, mice, rats, and humans, have high titers of natural antibodies against B-G polymorphic determinants. The specificity of this high background response has been studied by fusing lipopolysaccharide-stimulated spleen cells from unimmunized mice with myeloma cells and screening the hybrids for the production of antibody against chicken RBC (CRBC) (12). Clones specific for CRBC represented >1% of the total hybridomas and >95% of them secreted antibody against polymorphic CRBC determinants. Based on these and other observations, a model for the advantages of genetic polymorphism as a protection

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Abbreviations used in this paper: CAM, chorioallantoic membranes; CRBC, chicken erythrocytes; FP*, chimeric FP birds; GVH, graft-vs.-host; MHC, major histocompatibility complex; PBL, peripheral blood lymphocytes; RBC, erythrocytes; SC*, chimeric SC birds.
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against antigen mimicry by parasites has been proposed (12, 13). One of the predictions of the model is that the presence of natural antibody against polymorphic determinants of other individuals of the same or different species results from priming by cross-reactive bacterial antigens (14-17). Such natural antibodies may prevent bacterial infections (12) or serve as a protection against the development of cancer (18, 19). Because polymorphic determinants on B-G antigens may be important cross-reactive determinants, against which a significant proportion of the B cells in a mouse spleen might be directed, an understanding of their chemical nature, immunogenicity, and tolerogenicity might give important clues regarding the functional significance of polymorphism of cell surface antigens.

Recent evidence strongly suggests that B-G antigens are highly immunogenic targets for humoral immunity (10) and also for the production of antigen-specific T cell helper factor in the mouse (20). Thus, it is probably important to maintain a profound state of tolerance to such potentially immunogenic determinants, which may be cross-reactive with environmental antigens, as even a low level immune attack on them may lead to disastrous autoimmune reactions. In this paper, we show that there is a critical period in embryogenesis during which the induction of allogenic erythrocytic chimerism leads to the development of severe autoimmune anemia and lymphomas in adult life, and evidence is presented that the immune response to B-G determinants is responsible for the pathological events that follow.

Materials and Methods

Animals. Fertilized eggs of SC and FP strain chickens were purchased from Hy-Line International, Johnston, IO. These eggs are hybrids between different inbred strains of white Leghorn chickens. The SC and FP birds carry the B/B^2 and B^14/B^14 MHC alleles, respectively. All chickens were hatched and raised in the facilities of the University of Alberta Edmonton, Alberta, Canada. Fertile eggs from randomly bred white Leghorn chickens were obtained from the University of Alberta poultry research farm. Fertile eggs from B^14/B^14, B^14/B^15, B^14/B^13, and B^11/B^21 strains were also used for GVH assays. These strains are maintained in the poultry breeding facilities of the University of Alberta.

Production of Chimeras. Two different methods for generating hematopoietic chimeras using the SC and FP strains were used in the study.

For parabiosis, two 12-d-old embryos of the two different known genotypes (SC and FP) were joined together through their chorioallantoic membranes (CAM) and sealed with paraffin wax. At day 12 of incubation, windows were marked on the midpoint of the long axis (as described later for splenomegaly) over a forked vein. The eggshell and the shell membranes were then removed so that the CAM were fully uncovered. Immediately thereafter, the two genetically different eggs were joined together by their exposed CAM using paraffin wax, and then returned to the incubator. Care was taken to turn the parabionts at least once daily. Close to the time of hatching, the parabionts were put in nylon stockings and separated from each other with masking tape, in such a way that each chick hatched in different compartments of the nylon stocking.

Hematopoietic chimeras were also generated by stem cell transfer. The source of the hematopoietic stem cells was the embryonic spleen. Routinely, 30 pooled spleens of 15-, 17-, or 21-d-old SC or FP embryos were used as the donor cell population. Spleens were minced carefully in 10 ml RPMI 1640 serum-free medium until single cell suspensions were obtained. The cells were then allowed to settle for 20 min through 10 ml of medium in 12-ml test tubes. The supernates, which contained the hematopoietic stem cells (21), were harvested. The cell concentration was adjusted so that 1.2 × 10^7 non-RBC spleen cells were injected intravenously into 15-d-old SC or FP embryos. The same number of 17- or 21-d-old spleen cells were, in some experiments, also administered either intravenously to 17-d-old SC embryos or intraperitoneally to newly hatched SC chicks.
Specific alloantisera were produced by reciprocal immunizations between strains SC and FP. Each bird received three injections each of 2 ml of citrated adult whole blood every 2nd d. 6 d after the last injection, the hemagglutination titer was determined. If the titer was lower than 1:32, the above immunization procedure was repeated after a 2-wk rest period. As the strains used are not inbred for all non-B loci, the immune sera produced were extensively absorbed with erythrocytes from many different members of the host strain. Absorption was carried out at room temperature for 30 min using equal volumes of antiserum and packed RBC. The procedure was repeated three times. Finally, the serum was aliquoted in small volumes and stored at -30°C.

Detection of Chimerism. All birds generated by the two methods described previously were tested for RBC chimerism using the haplotype-specific anti-B locus sera, produced as described above. Two methods were used for determining the extent of chimerism.

The RBC agglutination method was the assay routinely used for a qualitative and semi-quantitative estimate of chimerism. Before typing the putative chimeras, the strength of the antiserum was characterized by typing different mixtures of RBC from the two strains used. Generally, a 10% chimerism was easily detected, and a strong antiserum could often detect as little as 5% chimerism.

The cellular radioimmunoassay was also used; details on this assay have been published elsewhere (22). Briefly, \(10^7\) RBC cells from the putative chimeras were incubated with 0.1 ml of specific alloantiserum for 30 min at room temperature. Then the cells were washed four times and further incubated with 0.2 ml of \(^{125}\)I-rabbit anti-chicken immunoglobulin (Ig). After incubation for 30 min at room temperature, the washing procedure was repeated and the cells were harvested and counted. The degree of chimerism was estimated by comparing the counts found with those from artificial mixtures.

GVH Reactions. Cell-mediated responsiveness was analyzed by two manifestations of GVH reactivity, namely, the splenomegaly and the CAM-pock reactions.

For the splenomegaly reaction, the procedure was as has been described previously (23). The reaction involves spleen enlargement as measured by increment in weight that results from the intravenous injection of immunocompetent lymphocytes into 12-d-old embryos measured 4 d postinjection. The assay is known to measure alloreactivity resulting from major B complex differences.

Cell-mediated responsiveness was also measured by the CAM-pock reaction. This involves the formation of pocks (lesions of characteristic morphology and size) after inoculation of donor immunocompetent lymphocytes onto the CAM of 12-d-old embryos differing from the donor lymphocytes at the B locus. Pocks are enumerated 4 d postinoculation (24).

Donor Cell Preparation. Preparation of peripheral blood lymphocytes (PBL) was as follows: Heparinized (10 U/ml) peripheral blood was obtained from appropriate donors. The blood was then distributed in 5-ml culture plastic tubes (4 ml/tube) and centrifuged at 600 rpm for 6 min. The lymphocyte-rich plasma supernatant was collected while avoiding any RBC contamination. The lymphocytes were washed twice in RPMI 1640 by centrifugation at 1,200 rpm for 6 min. Finally, the cells were resuspended, and viable counts were made using trypan blue dye.

For spleen cell preparation, donor spleens were removed under sterile conditions and minced in RPMI 1640 until a single cell suspension was obtained. Cell clumps were allowed to settle out for 15 min under gravity in 15-ml culture plastic tubes (~10 ml/tube) and the single-cell-containing supernatant was collected. The cells were washed twice and counted as before.

Results

Hematopoietic chimeras were produced by administering embryonic spleen cells to allogeneic chick embryos at different stages of embryogenesis. The immunological and pathological characteristics of these chimeras differed dramatically depending on the stage of embryonic development at which they were generated.

Long-term GVH Tolerance of Chimeras Generated at Days 12 and 15 of Embryonic Development. Chimerism induced by parabiosis of day 12 embryos was stable and was found to persist for the lifetime of all animals tested. This is in agreement with other
published data (25). All seven birds tested for erythroid chimerism at 120 wk of age were found to be between 10 and 50% chimeric for donor-type erythrocytes. Successful chimerism was similarly induced at day 15 of embryogenesis by intravenous injection of embryonic spleen cells. All 13 birds of this group have been shown previously to be chimeric for FP type erythrocytes (22). The degree of success in generating day 15 chimeras in three different groups was 100%, 85%, and 80% (data not shown). A bird judged to be chimeric had at least 5% of donor type erythrocytes, and only these birds were included in the analysis.

Two types of GVH assays were used to assess T cell-mediated immunity against MHC alloantigens: the GVH splenomegalgy reaction and the CAM-pock reaction. All experimental birds used in these studies were erythroid chimeras at the time of GVH testing.

Table I shows the results obtained when lymphocytes from eight FP birds were tested individually for their ability to cause GVH splenomegalgy reactions. In experiment 1, lymphocytes from three 20-wk-old chimeric FP birds (FP*), generated at day 15 of embryogenesis, were used to assay for GVH splenomegalgy, as described in Materials and Methods.

### Table I

**GVH Reactivity* of Adult Chimeric Birds, Generated at Days 12 and 15 of Embryogenesis, as Assayed by Splenomegalgy**

| Source of donor cells | Weight of spleens 4 d postinjection | SC hosts‡ | Outbred hosts |
|-----------------------|-----------------------------------|------------|---------------|
|                       | mg                                |            |               |
| **Experiment 1§**     |                                   |            |               |
| FP* 1                 | 12.0 ± 0.4 (a)                     | 78.5 ± 14.6|               |
| FP* 2                 | 12.4 ± 0.6 (a)                     | 58.5 ± 13.4|               |
| FP* 3                 | 11.3 ± 0.4 (a)                     | 81.2 ± 16.7|               |
| FP normal control I   | 70.8 ± 8.2 (b)                     | 127.8 ± 25.9|               |
| **Experiment 2¶**     |                                   |            |               |
| FP* 1                 | 10.5 ± 0.4 (a)                     | 60.8 ± 21.5|               |
| FP* 2                 | 10.5 ± 0.5 (a)                     | 85.0 ± 50.0|               |
| FP* 3                 | 11.6 ± 0.5 (a)                     | 65.6 ± 32.2|               |
| FP* 4                 | 9.3 ± 0.6 (a)                      | 52.2 ± 13.4|               |
| FP* 5                 | 9.1 ± 0.5 (a)                      | 69.0 ± 24.2|               |
| FP normal control II  | 74.0 ± 62 (b)                      | 134.0 ± 25.0|               |
| SC normal control II  | 11.2 ± 0.6 (a)                     | 147.0 ± 37.5|               |

* Whole blood was washed twice in RPMI 1640 serum-free medium. 0.1 ml of 1:3 reconstituted whole blood was used in both experiments to assay for GVH splenomegalgy, as described in Materials and Methods.

‡ 9-12 host embryos were used per experimental group. Results are shown as mean ± SE. Groups (a) and (b) of experiments 1 and 2 differ significantly (P < 0.001).

§ Three FP* birds, 20 wk of age, made chimeric with SC after parabiosis at day 12 of embryogenesis.

¶ FP and SC control birds used in these experiments were age-matched birds that had been injected at the 15th d of embryogenesis with syngeneic embryonic stem cells.

§ Five FP* birds, 7 wk old, made chimeric with SC after stem cell infusion at day 15 of embryogenesis.
12 of embryogenesis by parabiosis, were tested for their ability to cause splenomegaly when injected into embryos of the donor-type strain or into third-party outbred embryos. In experiment 2, lymphocytes from five FP birds made chimeric (FP*) at day 15 of embryogenesis, were also examined for their ability to cause splenomegaly. As can be seen, complete tolerance was induced in both types of experimental birds. The reaction caused by the injection of lymphocytes from FP* birds into SC embryos was identical with that obtained when SC embryos were injected with adult SC lymphocytes. The specificity of the reaction was shown by the ability of lymphocytes from chimeric birds to cause GVH splenomegaly when injected into outbred 12-d-old embryos. When lymphocytes from normal and tolerant birds were compared for their ability to cause GVH reactivity after injection into outbred embryos, it was found that the tolerant lymphocytes showed a lower reactivity. This is most likely a result of the induction of cross tolerance.

Table II shows the results obtained when lymphocytes from eight chimeric SC (SC*) birds were tested individually for their ability to induce GVH pocks. In this table, chimeras generated at day 12 parabiosis and day 15 after stem cell injection are included in two different experiments. The CAM-pock assay used in these experiments enumerates alloreactive lymphocytes that are placed onto the CAM of allogeneic

| Source of donor cells | Number of pocks 4 d postinoculation |
|----------------------|-------------------------------------|
|                      | Mean ± SE                           |
| FP hosts‡            |                                     |
| Experiment 1§        |                                     |
| SC* 1                | 0 (7) (a)                           | 12.0 ± 6.6
| SC* 2                | 0 (8) (a)                           | 12.6 ± 4.2
| SC* 3                | 0 (6) (a)                           | 10.2 ± 3.4
| SC normal control    | 22.7 ± 6.4 (b)                      | 31.5 ± 9.4
| Experiment‖          |                                     |
| SC* 1                | 0 (8) (a)                           | 55.0 ± 9.6
| SC* 2                | 0 (7) (a)                           | 22.5 ± 8.7
| SC* 3                | 0 (8) (a)                           | 24.8 ± 11.4
| SC* 4                | 0 (7) (a)                           | 24.0 ± 8.6
| SC* 5                | 0 (8) (a)                           | 18.7 ± 6.5
| SC normal control    | 43.5 ± 5.5 (b)                      | 56.1 ± 13.0

* Whole blood was washed twice in RPMI 1640 serum-free medium. 0.1 ml of 1:3 (experiment 1) or 1:2 (experiment 2) reconstituted whole blood was used in both experiments to assay for GVH CAM-pock reactivity as described in Materials and Methods.
‡ 9-12 host embryos were injected per experimental group. The number of CAM recovered is given in parenthesis. Groups (a) and (b) of experiment 1 and 2 differ significantly (P < 0.001).
§ Three SC birds, 16 wk old, made chimeric with FP after parabiosis at day 12 of embryogenesis.
‖ Five SC birds, 20 wk old, made chimeric with FP after stem cell infusion at day 15 of embryogenesis.
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embryos. Pocks of characteristic morphology and size are formed on the CAM of the embryos within 4 d when the donor immunocompetent lymphocytes differ from the embryos at the MHC region. The CAM-pock assay is, therefore, a very sensitive technique in assessing the degree and specificity of tolerance. As can be seen in Table II, complete and specific tolerance was induced in both types of chimeras as assayed by this GVH reaction.

A total of 91 SC* and 29 FP* birds were generated at day 12 or day 15 and tested individually in different experiments over a period of 4 yr. Complete and specific tolerance was found to be a permanent property of most of these birds that remained chimeric over a long period of time.

Specific Absence of Alloantibodies, Natural or Induced, in Chimeras Generated at Days 12 and 15 of Embryogenesis. Two approaches were used to analyze the state of responsiveness, at the humoral level, toward donor RBC-associated MHC alloantigens of chimeras made at days 12 and 15 of embryonic development. The first was the measurement of the specificity of preexisting, natural anti-RBC throrocyte alloantibody (10, 11) in the serum of otherwise untreated chimeras. The second was the measurement of alloantibody in the serum of chimeras that had been immunized with donor blood under conditions that are known to elicit a good alloantibody response in normal birds.

Sera from normal adult birds contain natural antibodies against B-G alloantigens of the species, other than those expressed as self antigens (10, 11, and B. M. Longenecker, unpublished data). A more sensitive, indirect agglutination technique is required to reveal these antibodies, as direct hemagglutination is never found using these sera. The results shown in Table III demonstrate that all 10 SC* chimeras tested had natural antibodies against third-party B\(^{19}/B^{19}\) RBC, but only 2 of 10 demonstrated natural antibodies against donor-strain RBC. These two positive sera probably represent the early appearance of induced anti-donor-strain alloantibodies, which occurs in a minority of day 15 chimeras and which will be described below.

The second approach was to intentionally immunize a group of SC* chimeras, generated at day 15, with donor-type FP adult RBC mixed with RBC from third-party B\(^{14}/B^{14}\) donors. The sera obtained at day 12 after immunization, using established protocols for making alloantibodies (26), contained alloantibodies reacting with B\(^{14}/B^{14}\) RBC, but no donor strain anti-FP alloantibodies were detected (Table IV). Thus, these chimeras maintained a high level of humoral tolerance to both B-G and B-F MHC antigens expressed on donor-strain RBC.

Immunological Properties of Chimeras Generated at Day 17 of Embryogenesis. Table V shows the immunological properties of nine SC* chimeric birds tested at 7 wk of age.

| Table III |
| Natural Antibodies in the Sera* of SC* Chimeras Generated at Day 15 of Embryogenesis |

| Indirect agglutination of RBC from | SC(B\(^{2}/B^{2}\)) | FP(B\(^{19}/B^{19}\)) | B\(^{19}/B^{19}\) |
|----------------------------------|-----------------|-----------------|-----------------|
| O/10                             | 2/10            | 10/10           |

* From 6-wk-old chimeras.
‡ As described in Longenecker et al. (10).
¶ Number of positive sera per number tested.
TABLE IV

**Alloantibodies in SC Normal and Chimeric Birds after RBC Immunization***

| Group Immunization with | Direct agglutination~ on RBC from |
|------------------------|----------------------------------|
|                        | **FP** | **B₁₁/B₄** |
| SC normal FP RBC       | 5/5    | NT§       |
| SC* FP and B₁₁/B₄      | 0/12   | 12/12     |
| SC* B₁₁/B₄ RBC         | 0/4    | 4/4       |

* 12-wk-old birds were injected intravenously three times on alternate days with RBC from a total of 8 ml of blood from age-matched donors. RBC agglutination was tested 10-12 d after the first injection.  
~ Agglutination titers ranged from 1:8-1:64 in positive sera and <1:2 in negative sera.  
§ Not tested.

| TABLE V |

**Immunological Properties of Chimeras Generated at Day 17 of Embryogenesis**

| Source of donor lymphocytes and sera | Chimerism§ | CAM-pocks enumerated 4 d after lymphocyte inoculation on | Direct agglutination‡ titers measured on RBC of |
|-------------------------------------|------------|---------------------------------------------------------|-----------------------------------------------|
|                                     |            | (mean ± SE)                                             | (FP (B₁₁/B₄)) | (SC (B₁₁/B₄)) |
| SC normal control                   | 0          | 51.3 ± 6.9                                             | 0                | 0               |
| SC* 1                               | 10-20      | 0 (8)                                                   | 12.3 ± 4.6      | 0               |
| SC* 2                               | 10-20      | 0 (7)                                                   | 9.5 ± 3.5       | 0               |
| SC* 3                               | 20         | 0 (8)                                                   | 22.4 ± 4.9      | 1/2             |
| SC* 4                               | 10-20      | 0 (8)                                                   | 16.6 ± 4.6      | 3/8             |
| SC* 5                               | 10-20      | 0 (6)                                                   | 27.4 ± 15.6     | 0               |
| SC* 6                               | 30         | NT                                                      | NT               | 0               |
| SC* 7                               | 10         | NT                                                      | NT               | 0               |
| SC* 8                               | 20         | NT                                                      | NT               | 1/2             |
| SC* 9                               | 30         | NT                                                      | NT               | 3/8             |

* Lymphocytes from 7-wk-old SC birds, made chimeric at day 17 of embryogenesis, were tested for ability to cause GVH and CAM-pock reactions upon inoculation onto appropriate host embryos, as indicated. The sera from the same birds were tested for their ability to agglutinate RBC of the donor- or host-type strain.  
‡ Direct RBC agglutination titers were never found in numerous control birds.  
§ Chimerism in this table of experimental control birds was estimated by RBC agglutination as described in Materials and Methods.  
¶ 9-12 host embryos were used per experimental group. The number in parentheses represents the number of recovered membranes.  
† Not tested.

Successful erythrocytic chimerism was induced in all members of this group and ranged from ~10 to 30%. Lymphocytes from five randomly chosen birds showed complete and specific GVH tolerance when tested with donor type embryos. (In another experiment [data not shown], six randomly chosen birds showed the same results.) However, in contrast with the chimeras generated at day 12 or day 15 of embryogenesis, about one-half of the day 17 chimeras produced anti-donor-strain
alloantibodies detectable at 7 wk of age. By 8–10 wk, all chimeras tested were found to have alloantibodies that reacted specifically with donor-type RBC (Table VI). Birds that made anti-donor RBC alloantibodies nevertheless remained specifically tolerant to donor-embryo GVH alloantigens. To test whether these alloantibodies might also react with donor-type lymphocyte (B-F) alloantigens, the sera were tested for their capacity to inhibit GVH reactivity of donor-type lymphocytes. This inhibition of GVH splenomegaly is a very sensitive assay for anti-B-F (8) as well as for alloantibodies against other T lymphocyte alloantigens (27). None of the sera from the day 17 chimeras was able to inhibit GVH splenomegaly, although these sera had hemagglutinating alloantibodies against donor-type RBC (Table VII).

**Development of Autoimmunity and Lymphomas in Day 17 Chimeras.** 83% of the chimeric

### Table VI

**Specificity of Serum Alloantibodies from Nonimmunized SC* Chimeras Generated at Day 17 of Embryogenesis**

| Direct agglutination* on RBC from | SC (B²/B³) | B¹⁹/B¹⁹ | B¹³/B¹³ | B¹⁴/B¹⁴ | B¹⁵/B¹⁵ | FP (B¹⁵/B¹³) |
|----------------------------------|------------|---------|---------|---------|---------|-------------|
|                                  | 0/6        | 0/6     | 0/6     | 0/6     | 6/6     | 6/6         |

* Sera from six different chimeric birds, 8–10 wk old, were tested with the RBC of different genotypes.

### Table VII

**Absence of GVH Inhibition Activity in the Sera of Chimeric Birds That Have Antibodies against RBC of the Donor-type Strain**

| Source of 10⁶ donor lymphocytes | Type of serum | Agglutination titer of serum tested on RBC of | Weight of spleens 4 d after injection (mean ± SE)* |
|---------------------------------|---------------|---------------------------------------------|-----------------------------------------------|
|                                 |               | FP (B¹⁵/B¹³) | SC (B²/B³) | FP (B¹⁵/B¹³) | SC (B¹³/B¹³) |
| SC normal                       | SC normal     | 0           | 0           | 115.0 ± 13.6 |
| SC normal                       | SC anti-FP‡   | ½⁸          | 0           | 119.0 ± 24.0 |
| SC normal                       | SC* §         | ½⁸          | 0           | 126.0 ± 13.5 |
| SC normal                       | SC* §         | ¼           | 0           | 127.0 ± 15.7 |
| SC normal                       | SC* §         | ½²          | 0           | 112.1 ± 17.9 |
| SC normal                       | SC* §         | ¼           | 0           | 105.7 ± 16.3 |
| SC normal                       | SC* §         | ½⁶          | 0           | 120.2 ± 6.7 |
| FP normal                       | SC normal     | 74.3 ± 9.5  |             |               |
| FP normal                       | SC anti-FP    | 17.5 ± 2.1  |             |               |
| FP normal                       | SC* 1         | 69.0 ± 5.2  |             |               |
| FP normal                       | SC* 2         | 83.5 ± 6.1  |             |               |
| FP normal                       | SC* 3         | 77.5 ± 6.7  |             |               |

* 10⁶ purified PBL were incubated for 1 h at room temperature in 1 ml of 50% serum obtained from various donors as indicated. After washing once, these cells were injected into appropriate hosts and their GVH reactivity measured 4 d later by weighing the excised spleens of the hosts.

‡ The SC anti-FP serum tested was obtained after immunization of SC birds with FP whole blood and subsequently absorbing the serum with SC cells.

§ Sera from 8–10-wk-old chimeric birds.
birds generated at day 17 and a minority (~15%) of day 15 chimeras eventually
developed severe, antibody-mediated hemolytic anemia. An early sign of the disease,
which occurred after 16 wk of age, was the loss of color of their normally red combs.
The RBC from these birds were found to be strongly Coombs's positive after the
addition of rabbit-anti-chicken Ig. Sera from these birds also contained antibodies
that react with lymphocytes from both host- and donor-type antigens, also demonstrat-
ing an autoimmune state (Table VIII). When heavily diseased birds were bled,
their RBC showed signs of spontaneous agglutination and the presence of large
numbers of erythroblasts. Chimerism was undetectable at this time and they were
severely immunosuppressed as judged by the lack of general GVH reactivity (data
not shown). 100% of the diseased birds died by 10 mo of age. On autopsy, splenic
atrophy and extensively metastasizing lymphomas were found in the majority of these
birds. Large bursal tumors were always found, suggesting a B cell origin of the
lymphomas.

Chimeras Generated at Hatching (Day 21 of Embryonic Development). Chimeras
were produced at day 21 by intraperitoneal injection of spleen cells from either day 15
embryos or day 21 hatched chicks. In both groups, successful chimerism ranging from
10 to 30% was achieved in the majority of birds (85%) when tested at 3 wk of age
(Table IX). These birds, although chimeric for donor RBC, displayed normal GVH
reactivity against donor alloantigens when tested at 3 wk of age (Table IX). The same
birds, when tested at 16 wk of age, had lost detectable chimerism and maintained
GVH reactivity against donor alloantigens. Only 1 out of 22 birds tested developed
detectable anti-donor-strain RBC alloantibodies, and all birds remained healthy until
they were killed.

Discussion

Table X summarizes the immunological and pathological characteristics of the
RBC chimeras generated at different stages of embryonic development. Although
chimeras generated at different times displayed similar degrees of RBC chimerism,
which ranged from 5 to 40%, they showed vastly different immunological and
pathological characteristics. All chimeras generated at day 12 and most chimeras

| Source of serum | Lympocytes* of |
|-----------------|---------------|
|                 | SC (B²/B⁶)    | FP (B¹⁵/B¹⁷) | B²/B¹⁷ | B¹²/B¹³ |
| SC control      | 367 ± 17      | 1,073 ± 49   | 436 ± 26 | 715 ± 122 |
| SC* 1           | 2,587 ± 366   | 9,075 ± 160  | 5,802 ± 1,392 | 3,508 ± 376 |
| SC* 2           | 2,548 ± 260   | 6,448 ± 576  | 4,306 ± 488 | 3,736 ± 282 |
| SC* 3           | 3,158 ± 348   | 7,188 ± 1,056 | 4,075 ± 847 | 4,095 ± 519 |

* 10⁷ PBL from birds of different genotypes were incubated for 45 min with 0.1 ml of
a 1:10 dilution of test sera, the cells were washed, and then incubated with 0.1 ml of
¹²⁵I-labeled rabbit anti-chicken Ig. After rewashing, the cells were harvested and
counted in a gamma counter.

‡ Cell pellet.
**Table IX**

**Immunological Characteristics of Chimeras Generated at Day 21 of Embryogenesis**

| Source of donor lymphocytes | Properties examined at | 3 wk of age | 16 wk of age |
|----------------------------|------------------------|-------------|-------------|
|                            | RBC* chimerism | CAM-pocks formed on FP RBC | Direct agglutination titers measured on FP RBC | RBC chimerism | CAM-pocks formed on FP RBC | Direct agglutination titers measured on FP RBC |
|                            | % | mean ± SE | % | mean ± SE |
| SC normal                  | 0 | 54 ± 11.3 | 0 | 0 | 53.2 ± 12.1 | 0 |
| SC* 1†                    | 30 | NT | 0 | 0 | 43.1 ± 9.6 | 0 |
| SC* 2                    | 30 | NT | 0 | 0 | 53.2 ± 7.6 | 0 |
| SC* 3                    | ND** | 36.3 ± 8.0 | 0 | 0 | 45.4 ± 8.4 | 0 |
| SC* 4                    | 20 | 43.1 ± 5.3 | 0 | 0 | 42.2 ± 14.6 | 0 |
| SC* 5                    | 10 | 44.6 ± 8.6 | 0 | 0 | 45.7 ± 3.4 | 0 |
| SC* 6                    | 30 | 56.2 ± 7.5 | 0 | 0 | NT | 0 |
| SC* 7                    | 20 | NT | 0 | 0 | 48.3 ± 8.6 | 0 |

* Chimerism was estimated by both RBC agglutination and by the radioimmunoassay as described in Materials and Method.
† The ability of 10^6 PBL to cause GVH reactions was measured by counting pocks on the CAM of FP embryos 4 d after inoculation.
§ The ability of 1:3 washed and reconstituted whole blood to cause GVH reactions was measured by counted pocks on the CAM of FP embryos 4 d after inoculation.
|| SC chimeras were generated at hatching by intraperitoneal injection of spleen cells either from 15-d-old FP embryos or from 21-d-old newly hatched chicks.
¶ Not tested.
** Not detectable.

**Table X**

**Immunological and Pathological Characteristics of Avian-hematopoetic Chimeras Generated at Different Stages of Embryonic Development**

| Chimeras made at day | RBC chimerism | Number of birds tested | Proportion of birds with | Specific anti-donor GVH | Specific anti-donor antibody | Hemolytic anemia, lymphomas |
|----------------------|---------------|------------------------|--------------------------|------------------------|----------------------------|----------------------------|
|                      | %             |                        |                          |                       |                           |                            |
| 12*                  | 10-40         | 8                      | 0/8                      | 0/8                   | 0/8                       | 0/8                       |
| 15‡                  | 5-40          | 36                     | 0/36                     | 8/36                  | 6/36                       |                            |
| 17§                  | 10-30         | 22                     | 0/22                     | 13/22                 | 18/22                      |                            |
| 21†                  | 10-30 at 3 wk | 22                     | 22/22                    | 1/22                  | 0/22                       |                            |

0 at

>16 wk

* By parabiosis.
‡ By intravenous injection of 15-d-old allogeneic embryonic stem cells.
§ By intravenous injection of 17-d-old allogeneic embryonic stem cells.
† By intraperitoneal injection of 15-d-old allogeneic embryonic stem cells.
generated at day 15 were classic hematopoietic chimeras (25) in the sense that they maintained profound long-term chimerism and specific tolerance for all donor MHC alloantigens (B-F, B-L, and B-G) at both the humoral and cell-mediated level. The apparent absence of natural anti-donor strain antibody measured by indirect hemagglutination in these chimeras was of particular interest. Normal adult chickens as well as humans, mice, rats, and alligators have been shown to possess natural antibodies against polymorphic determinants on B-G region-encoded antigens expressed on CRBC (10-13). Normal adult chickens contain natural antibodies against B-G antigens of the species with the exception of their own B-G antigens. Most of the healthy adult chimeras were also found to lack natural antibodies against donor-strain B-G antigens. In this respect, the immunological properties of these chimeras with respect to donor antigens were identical to those of normal birds with respect to their own B-G antigens. If natural antibodies against B-G as well as RBC blood group antigens on CRBC are protective and arise because of infection with cross-reactive bacteria (10-13, 28), it might be predicted that chickens made chimeric and tolerant of many different B-G specificities might be more susceptible to certain types of bacterial infections.

Chimeras produced at a later stage of embryonic development, that is at day 17, displayed specific tolerance for GVH reactivity, but a high proportion of them developed alloantibodies against donor-strain RBC. At the same time, no antibodies against donor strain lymphocytes were detected using a sensitive GVH-inhibition assay. This assay is known to be especially sensitive for the detection of anti-B-F alloantibodies (8). B-G and B-F antigens are both well expressed on RBC (13). Nevertheless, our data strongly suggest that tolerance to B-F is maintained even though B-G tolerance has spontaneously broken. The appearance of specific anti-B-G alloantibodies was the earliest detectable sign preceding the development of autoimmune. These antibodies were apparently ineffective at eliminating chimerism. This was followed by the development of severe Coombs's-positive anemia, the development of anti-lymphocyte autoantibodies, severe immunosuppression, and the development of extensively metastasizing lymphomas. It seems likely that autoimmunity developed because the early anti-B-G alloantibodies plus the continuous presence of donor-type RBC in high proportion led to the production of anti-host antibodies, possibly as a result of the presence of donor B-G antigens that cross-react with host B-G specificities (29). This process could lead to the development of severe antibody-mediated hemolytic anemia. Examples are reported in the literature in which autoimmunity can be induced by chronically immunizing with antigens that cross-react with self. For example, autoimmunity is known to be induced in mice after prolonged immunization with xenogenic RBC (30) or with malaria-infected syngeneic RBC (31).

The autoimmune birds described in this paper developed massive lymphoid tumors. The association between generalized autoimmunity and lymphoid neoplasms also exists in individual patients (32) and in NZB mice. NZB mice develop autoantibodies against thymus cells when they are 1 mo old (33) and subsequently develop anti-RBC, anti-DNA, anti-RNA, and anti-G antibodies (Gross-type murine leukemia virus-related antigen). 25% of these mice develop lymphoid neoplasia (34). It appears that the experimental system described in chickens resembles, in many ways, the naturally occurring autoimmune disease in NZB mice.

Our results show that autoimmunity and malignancy can be experimentally
induced during embryogenesis. The lymphomas observed in the current work were similar to those of avian leucosis, which is a neoplasm of the B cell system induced by an RNA tumor virus in chickens (35). It is possible that chronic stimulation of the B cell system led to the activation of the virus and the induction of lymphomas. The development of B cell lymphomas during chronic GVH disease has been described in mice (36) where it was suggested that abnormal induction of donor T cells on autoreactive F1 B cells can initiate the production of autoantibodies and that chronic antigenic stimulation eventually leads to uncontrolled proliferation and the development of lymphomas (37). It is not clear whether the pathological process observed here involves a classic GVH reaction, but the development of bursal tumors might suggest that the B cell system received the chronic antigenic stimulation which led to the development of malignancy.

Attempts to induce GVH tolerance at hatching failed, although successful RBC chimerism was temporarily produced. Similar results were obtained when donor spleen cells for chimerism induction were obtained from day 15 embryos or newly hatched chicks. This shows that RBC progenitors exist in the newly hatched spleen despite the apparent presence of a lymphoid microenvironment and the absence of detectable erythroid microenvironment (21). It is of interest that full GVH competence for donor-type MHC antigens was maintained in the presence of a high degree of RBC chimerism. This strongly suggests that the relevant MHC antigens for GVH stimulation in the chicken are not those expressed on erythrocytes (B-G or B-F), but like in mammalian systems, class II molecules (B-L antigens) are the strongest GVH stimulators.

An interesting picture that emerges for our studies is the existence of a period during embryonic development, sometime between days 15 and 21, when the embryo is particularly susceptible to the induction of autoimmunity and lymphomas after the infusion of allogeneic stem cells. The chick thymus becomes lymphoid at day 12 of embryogenesis (38). All of the chimeras produced by parabiosis at this time and the majority of the chimeras produced by stem cell infusion at day 15 remained healthy, specifically unresponsive, and chimeric for their entire life span. By these criteria, the immunological status of these birds with respect to donor antigens is equivalent to that of a normal animal with respect to its own antigens.

The finding that there are critical stages of embryonic development during which either tolerance or immunity can be induced is not new. Buxton (39) reported that the administration of killed Salmonella pullorum to chicken embryos leads to tolerance if the antigen is administered before the 16th d of embryogenesis. Administration of the antigen at later times of embryogenesis did not induce tolerance. The same conclusion was reached by Mitchison (40) using turkey RBC. Both authors accounted for the importance of the time at which antigen is administered as a result of the transfer of maternal antibodies from the yolk sac to the embryonic circulation that occurs on the 15th d of incubation. It is possible that the aforementioned natural alloantibodies to B-G antigens also appear in the embryo at around the 15th d of incubation. It is interesting that this coincides with the beginning of the period in which the embryo is susceptible to the induction of autoimmunity and lymphomas.

By 21 d of development, the chick once again developed resistance to the induction of autoimmunity and lymphomas and yet remained susceptible to the induction of RBC chimerism but refractory to GVH tolerance induction. There are similar
observations in mice. Attempts to induce transplantation tolerance in mice at birth were successful in only 8% of the animals, whereas 23% and 43% of the mice were tolerant when antigen was given at days 18 and 15 of gestation, respectively (41). This is not surprising, as cytotoxic killer precursor cells can be detected in the spleens of 2-d-old mice (42, 43). It is interesting, however, that the development of immunocompetence in the newly hatched chick, although apparently preventing the establishment of lymphocyte chimerism and GVH tolerance, nevertheless, does not prevent the successful establishment of RBC chimerism. This suggests that tolerance to RBC-associated MHC antigens (both B-G and class I [B-F] antigens) might be easier to establish and maintain than tolerance to lymphocyte-associated, class II (B-L) antigens. Despite this, by 16 wk, RBC chimerism was apparently eliminated with no evidence for the continuous development of alloantibody to donor RBC alloantigens. The mechanism whereby chimerism was eliminated in these birds might, therefore, involve cell-mediated immunity, perhaps directed against B-G antigens, which are known to be expressed on erythroid progenitor cells (1). This might contrast with the mechanism of elimination of RBC chimerism in the chimeras established at day 17, where anti-RBC alloantibody is produced and leads eventually to autoimmunity and lymphoma.

Summary

Hematopoietic chimeras were produced at four different stages of ontogeny between two allogeneic strains of chickens. All chimeras produced by parabiosis at day 12 of embryogenesis and the majority (83%) of the ones produced at day 15 by intravenous injection of allogeneic stem cells remained healthy, chimeric, and specifically tolerant at both the humoral and cell-mediated level throughout a long examination period. Chimeras generated at day 17 of embryogenesis demonstrated specific unresponsiveness at the cell-mediated level but produced specific anti-donor alloantibodies directed against erythrocyte-associated major histocompatibility complex (MHC) (B-G) antigens.

These chimeras and a minority (17%) of the chimeras generated at day 15 of embryogenesis developed severe antibody-mediated autoimmune hemolytic anemia after the 5th mo of age and succumbed to massive bursal lymphomas and metastases by the 10th mo of age. The immunological and pathological characteristics of these birds appear to reflect an autoimmune state rather than one of tolerance. Erythroid chimeras generated at day 21 of ontogenic development displayed normal levels of GVH reactivity. These birds were eventually able to eliminate the chimeric state and remained healthy until deliberately killed. These results show that there is a critical period in embryogenesis during which the induction of allogeneic erythrocytic chimerism leads to the development, in adult life, of severe autoimmune anemia, B cell lymphomas, and death.

B-G MHC antigens are erythroid differentiation antigens of the chicken. Polymorphic determinants on B-G antigens appear to be important cross-reactive determinants (with environmental bacteria), against which a high background immunity exists. Evidence is presented that the immune response to B-G antigens is responsible for the development of autoimmunity and other pathological events that follow and that
tolerance to class I MHC antigens (B-F antigens) shared by lymphocytes and erythrocytes is maintained at the same time that B-G tolerance is broken.

Received for publication 29 March 1982.

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