GENERATION OF IMMUNOGLOBULIN HEAVY CHAIN DIVERSITY SUBSEQUENT TO CELL SURFACE IMMUNOGLOBULIN EXPRESSION IN THE AVIAN BURSA OF FABRICIUS

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The generation of a pre-immune repertoire of B lymphocyte specificities is a prerequisite for subsequent antigen-dependent antibody responses. In avian species, the bursa of Fabricius plays a central role in the development of B lineage cells (1-3) and in the generation of antibody diversity (4-6). During embryonic life the bursa is colonized with B cell precursors (7, 8) that rearrange and express Ig genes early in bursal development (2, 9). Recent evidence has suggested that each of ~10^4 discrete follicles within the bursa (10) is colonized by two to four precursor cells, and so the total number of B cell precursors in the chicken is low, probably <5 x 10^4 (11). While a detailed analysis of the repertoire of peripheral B cell specificities in the chicken has not been made, the available evidence suggests that the avian B cell repertoire is not grossly less than that of mammalian species (12, 13). Thus, estimates have been made that a repertoire of at least 10^6 specificities is generated from <5 x 10^4 B cell precursors (14).

Cyclophosphamide (CY) destroys bursal lymphocytes (15) while maintaining the functional integrity of the bursal epithelium (16). The neonatal bursa contains a population of B cell precursors, termed bursal stem cells (busc), that can reconstitute the bursal lymphoid compartment of CY-treated recipients (16). Under conditions of limiting donor busc numbers, where fewer than ~30% of recipient bursal follicles are repopulated, the great majority of reconstituted follicles are clonal, each containing cells derived from a single busc (10).

The busc expresses surface Ig; its ability to reconstitute the bursa of CY-treated recipients is inhibited by pre-exposure to anti-Ig antibodies (11). In particular, treatment of busc from IgM-1 (Cμ) allotype heterozygous donors with antibodies to one or another IgM-1 allotype selectively inhibits the repopulation of CY-treated recipients with cells expressing that allotype but not by cells expressing the alternative IgM-1 allele (9, 11).

Each sIg+ busc must therefore express a particular Ig H chain V region at the time of cell transfer. In the absence of any Ig diversification subsequent to Ig expres-
sion, the progeny of any given bursa should all express identical Ig V regions and therefore be homogeneous for the expression (or not) of any V region idiotypic determinant. Conversely, the presence of idiotypic heterogeneity within clonal bursal follicles would demonstrate Ig diversification in the bursa subsequent to sIg gene expression.

In the experiments reported here, limiting numbers of donor bursa were used to repopulate the bursae of CY-treated recipients such that each reconstituted bursal follicle was colonized by a single bursa. The expression of an H chain V region idiotype among the progeny of cloned bursa is shown to be heterogeneous. This result proves that the generation of Ig H chain diversity in the bursa of Fabricius can occur subsequent to Ig gene expression on the surface of avian B cell precursors.

**Materials and Methods**

**Chimeras.** Neonatal WL x GC chickens (Covour, Mirabel, Quebec) were treated with 2 mg cyclophosphamide (Cytoxan; Bristol-Myers, Ottawa, Ontario) on four consecutive days and reconstituted with ~15 x 10^6 bursal cells from 4-d-old donors. The donor cells comprised an equal mixture of cells from Bu-I^-b and Bu-I^b^ donors. Donor cell chimerism was monitored by assaying the levels of Bu-la^- and Bu-Ib^- cells in peripheral blood. Chimeras were killed at 6-8 wk after reconstitution for analysis of spleen and bursa.

**Antibody Reagents.** The mAbs 21-1A4 (anti-Bu-la; reference 17) and Fu5-11G2 (anti-Bu-Ib; reference 17) were purified from tissue culture supernatants using an anti-mouse k sepharose column (187.1; reference 18) and conjugated directly to FITC. The mouse anti-chicken Ig L chain mAb 11C6.F12.D6 (11C6) was isolated and cloned from a fusion between Sp2/0.Ag14 and spleen cells from a BALB/c mouse immunized with chicken IgM. Purified 11C6 antibodies were prepared using an anti-mouse k sepharose column as before. The mouse anti-Cv,-1 mAb (19) was obtained and used as an Ig preparation of ascites. Biotin-conjugated goat anti-mouse Ig was purchased from Sigma Chemical Co., St. Louis, MO, and was absorbed on chicken bursal cells before use. R-phycocerythrin-conjugated streptavidin was purchased from Southern Biotechnology Associates, Birmingham, AL.

**Cell Surface Staining.** Viable cells from pieces of bursal tissue, containing ~500 follicles, or from spleens were prepared by centrifugation of cell suspensions over lymphocyte (Cedarlane, Hornby, Ontario). Cell suspensions from individually dissected bursal follicles were prepared from the bursae of 6-8-wk-old chimeras as described elsewhere (9, 11).

Cell suspensions were stained with anti-chicken L chain (11C6), anti-Cv,-1 or the irrelevant monoclonal LN190 (y1, k; M. H. Julius, unpublished results), or HO13.4 (y, k; reference 20), all at 10 μg/ml, followed sequentially by biotin-conjugated goat anti-mouse Ig, R-phycocerythrin-conjugated streptavidin, normal mouse serum to block any available sites on the anti-mouse Ig, and finally, FITC conjugates of either anti-Bu-la or anti-Bu-Ib. All staining was performed in Dulbecco’s PBS and cells were suspended for analysis in 10 μg/ml propidium iodide (Sigma Chemical Co.) to stain the DNA of nonviable cells. Samples were analyzed on a FACScan (Becton Dickinson & Co., Mountain View, CA), viable cells being analyzed by gating on forward scatter, side scatter, and the exclusion of cells stained with propidium iodide.

**Results**

The chicken B cell alloantigen Bu-1 is polymorphic with two codominantly expressed alleles, Bu-I^- and Bu-I^ (21) (detected by mAbs 21-1A4 and Fu5-11G2, respectively; reference 17), providing a convenient system to demonstrate the clonal nature of bursal reconstitution by bursa (11). CY-treated neonatal recipient chickens were reconstituted with a mixture of neonatal bursal cells containing bursa from Bu-I^- (Fig. 1 a) and Bu-I^ (Fig. 1 b) donors. Engraftment of donor cells was monitored by staining PBL for the presence of cells expressing Bu-la and/or Bu-Ib. CY-
Clonality of bursal follicle reconstitution by bursal stem cells. Staining with FITC-conjugated anti-Bu-la (solid lines) or FITC-conjugated anti-Bu-lb (dashed lines) of bursal cells from (a) Bu-1\(a^k\) donors, (b) Bu-1\(b^k\) donors, and (c) CY-treated recipients reconstituted with a mixture of bursal stem cells from Bu-1\(a^k\) and Bu-1\(b^k\) donors, or of cells from individual follicles (d-f) from such chimeras stained with FITC anti-Bu-la (d and e) or FITC anti-Bu-lb (f).

Cells in 24 chimeric clonal follicles were analyzed for the expression of sIg. All follicles contained high frequencies of sIg\(^+\) cells (e.g., Fig. 2, c and d) with profiles essentially indistinguishable from those of either reconstituted (e.g., Fig. 2 b), or normal bursae (Fig. 2 a) as a whole. No heterogeneity in sIg expression was therefore observed among clonal follicles.

The anti-chicken idiotypic mAb anti-CV\(_n\)-1 was raised against chicken Ig derived from the tumor 293B\(_b\). This antibody recognizes some but not all chicken Ig molecules of all isotypes and isolated 293B\(_b\) \(\mu\) H chains (19). Anti-CV\(_n\)-1 antibodies stained the 293B\(_b\) tumor with an intensity equivalent to that observed with 11C6.
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Figure 2. Expression of surface Ig in clonal follicles. Staining of bursal cells (a and b) or cells from individual chimeric follicles (c and d) with II6C6, anti-chicken Ig L chain (solid lines) or irrelevant antibodies (dashed lines). (a) Normal bursal cells; (b) bursal cells from a CY-treated bursa-reconstituted chimera; (c) individual chimeric follicle containing exclusively Bu-la' cells; and (d) individual chimeric follicle containing exclusively Bu-la' cells.

76 follicles from three chimeras were productively analyzed for the presence of cells expressing the CVH-1 idiotype (e.g., Fig. 3, c–f). Of these, 74 of 76 follicles contained between 4 and 46% of CVH-1+ cells (Fig. 4). The remaining two follicles did not contain significant numbers of CVH-1+ cells (<4%). There was no correlation between the percentage of CVH-1+ cells within a follicle and the expression of the Bu-la within that follicle. The expression of CVH-1 is therefore heterogeneous within follicles derived from single slg+ B cell precursors. Thus, the generation of Ig diversity can occur subsequent to Ig gene expression in the bursa of Fabricius.

Reconstitution of the periphery of CY-treated recipients with bursal cells from 4-d-old donors requires maturation of the donor cells through the bursa of the recipient. Reconstitution of surgically bursectomized recipients by such donor cells is ineffective (16). It was of interest to determine, therefore, whether the diversity generated in the bursa was expressed in the peripheral B cell compartment.

Spleens from the three chimeras shown in Fig. 4 contained 8, 13, and 19% Bu-lb' cells (e.g., Fig. 5 a). The FITC-stained Bu-lb' cells were essentially all slg+ (Fig. 5 b) and were examined for the expression of the CVH-1 idiotypes. 15-28% of spleen B cells (Bu-lb') expressed the CVH-1 idiotype (e.g., Fig. 5 c), similar to the proportions of CVH-1 idiotype-expressing chimeric bursal cells. Analysis of idiotype expression on cells expressing the Bu-la allele (i.e., cells derived from one of the two donor populations and therefore present at a lower frequency than cells expressing the Bu-lb allele) revealed no obvious segregation of idiotype frequency between the

(anti-L chain). In contrast, anti-CVH-1 did not stain the slg+ tumor II6S1, provided by Dr. E. H. Humphries (University of Texas Southwestern Health Sciences Center), above background (results not shown).

Anti-CVH-1 stained ~21% of normal bursal cells from 4-d-old donors (Fig. 3 a). Similarly, in the chimeras reported here, CVH-1 expression ranged from ~15-20% of bursal cells (e.g., Fig. 3 b).

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two donor populations in each chimera (results not shown). Thus, the diversity generated subsequent to Ig expression in the chimeric bursae becomes expressed in the peripheral B cell compartment.
Discussion

The bursa of Fabricius has long been recognized as the major, if not the only site of normal B cell lymphopoiesis in avian species. Surgical (3), hormonal (22), or chemical (15) ablation of the bursa or the lymphoid cells it contains can lead to persistent depletion of peripheral B cells. It has also been well established that the bursa represents a site for the generation of Ig diversity. Thus, bursal lymphocytes binding specific antigens first appear subsequent to the appearance of the first slg* cells in the bursa (5). Furthermore, when not leading to complete agammaglobulinemia, bursectomy can lead to reduced heterogeneity of circulating Ig's and antibody responses (4, 6).

Recent evidence has demonstrated that the number of B cell precursors productively colonizing each bursal follicle in a normal chicken is in the range of two to five (11). Furthermore, the number of productive rearrangement events from which the cells in a normal bursal follicle are derived is very similar (two to four; references 9 and 23). This suggests that Ig gene rearrangement is a very early event in the development of the B cell lineage and may moreover represent the limiting event in the productive colonization of the bursa by B cell precursors. The conclusion that all B cell precursors in the chicken express slg before hatching (9) suggests that the available Ig diversity in the periphery of the adult chicken is derived from cells expressing slg before hatching. In the experiments reported here, the generation of Ig H chain diversity is demonstrated to occur after slg expression. Thus, the generation of antibody H chain diversity in the pre-immune B cell repertoire is not restricted to the stage in B lineage development at which Ig gene rearrangement occurs.

The results presented here extend the observation of Ig L chain diversification, as judged by IEF, in the progeny of busc from clonally reconstituted follicles (24).
Taken together, these results suggest that the sIg* busc and at least some of its progeny retain the capacity for extensive somatic diversification.

Recent evidence has suggested that the mechanism by which Ig L chain diversity is generated may be distinct, in the chicken, from that of Ig gene rearrangement. All B cells appear to undergo the same Ig L chain rearrangement event, juxtaposing a single functional VL gene to a single JL segment (25). Subsequent to Ig gene rearrangement (26), sequences derived from an upstream family of pseudogenes replace sequences within the rearrangement V region gene (27). This process, which has been dubbed gene conversion (26, 27), provides a likely mechanism for the generation of Ig L chain diversity subsequent to Ig expression, i.e., in the progeny of the busc.

Chicken B cells have similarly undergone a limited number of different Ig H chain rearrangement events (23). Furthermore, a family of pseudogenes lies upstream of Cu (28). It therefore seems reasonable to suggest (28) that a similar mechanism of gene conversion subsequent to Ig gene rearrangement may generate Ig H chain diversity. The results presented here demonstrating Ig H chain diversification in the progeny of sIg* busc support such a model.

The bursal stem cell represents a small fraction of bursal cells (0.01–0.1% of neonatal bursal cells) that declines with time (16). There are as yet no reagents that allow the busc to be distinguished or separated from the majority of bursal cells. It is unknown, therefore, whether the busc represents a reservoir of B cell precursors that have undergone gene rearrangement but remain as yet undiversified.

The rate of Ig diversification of Ig H chains in the progeny of busc is sufficiently high enough to allow clonally reconstituted follicles to attain relative homogeneity one with another. As yet, no DNA sequence analysis is available to analyze the rate of putative Ig H chain gene conversion events. However, from published data (27), an estimate of the rate for Ig L chain diversification can be made. Ig gene rearrangement precedes gene conversion. Assuming gene rearrangement occurs at about day 12e, then at day 18e each sIg* cell will have undergone ~15 cell divisions (5). Expressed V region sequences from day 18e bursal cells have on average one to two gene conversion events impinging upon their rearranged L chain genes. This number may be an underestimate if not all gene conversion events result in a change of V region sequence. Since Ig diversification of H chains also occurs subsequent to Ig expression, as demonstrated in this report, it seems reasonable to suggest that such diversification may occur by a similar mechanism at a similar rate. Thus, in ~15 cell generations, a cell will have undergone on average at least three gene conversion events, i.e., one every five cell doublings. This high rate of diversification, taken together with the observation that a single gene conversion event often leads to multiple amino acid changes in the expressed V region gene (27), is consistent with the observed small foci of antigen-binding cells in cryostat sections of normal bursae (15).

The bursa of Fabricius represents a site of Ig diversification among a population of cells that express Ig and therefore have the potential to recognize either exogenous or self antigen. The role of antigen in the generation of the bursal lymphocyte repertoire of specificities or, possibly more importantly, in the positive or negative selection from the repertoire being generated, is currently unknown.

Summary

The avian bursa of Fabricius represents a site for the generation of antibody diversity. Transfer of neonatal bursal cells into cyclophosphamide-treated neonatal chickens
results in reconstitution of recipient bursae with donor-derived bursal stem cells. These stem cells express cell surface IgM and, under conditions of limiting donor bursal stem cell numbers, each reconstituted bursal follicle is colonized by a single precursor cell. The expression of an Ig V\_\alpha idiotypic, CV\_\alpha-1, was found to be heterogeneous within such clonal follicles. The diversity generated within the bursa is subsequently found within the peripheral B cell compartment. Thus, the generation of functional Ig H chain diversity is shown to occur subsequent to Ig H chain rearrangement and expression.

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