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Perinatal Deletion of B Cells Expressing Surface Ig Molecules That Lack V(D)J-Encoded Determinants in the Bursa of Fabricius Is Not Due to Intrafollicular Competition

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During embryonic development, the avian bursa of Fabricius selects B cell precursors that have undergone productive V(D)J recombination for expansion in oligoclonal follicles. During this expansion, Ig diversity is generated by gene conversion. We have used retroviral gene transfer in vivo to introduce surface Ig molecules that lack V(D)J-encoded determinants into B cell precursors. This truncated \( \mu \) heavy chain supports both B cell expansion within embryo bursal lymphoid follicles and gene conversion. We show that individual follicles can be colonized exclusively by cells expressing the truncated \( \mu \) chain and lacking endogenous surface IgM, ruling out a requirement for V(D)J-encoded determinants in the establishment of bursal lymphoid follicles. In striking contrast to their normal development in the embryo, bursal cells expressing the truncated \( \mu \)-chain exhibit reduced rates of cell division and increased levels of apoptosis after hatching. The level of apoptosis in individual follicles reflects the proportion of cells within the follicle that express the truncated \( \mu \)-chain. In particular, high levels of apoptosis are associated with follicles containing exclusively cells expressing the truncated \( \mu \) receptor. Thus, apoptotic elimination of such cells is not due to competition within the follicle by cells expressing endogenous surface IgM receptors. This provides the first direct demonstration that the regulation of B cell development in the avian bursa after hatching differs fundamentally from that seen in the embryo. The requirement for intact IgM expression when the bursa is exposed to exogenous Ag implicates a role for Ag in avian B cell development after hatching. The Journal of Immunology, 2000, 164: 5041–5048.

The bursa of Fabricius is the primary lymphoid organ responsible for B cell lymphopoiesis in birds (1–6). Although the bursal microenvironment is not required for Ig gene rearrangement (7, 8) or expression (9, 10), B-lineage-committed precursor cells migrate in a single wave to the bursal primordium during embryonic life (11, 12). Those precursors that undergo productive Ig gene rearrangement migrate across the basement membrane and form follicles of rapidly dividing cells (13, 14), termed epithelial buds, and it is from these cells that the entire B cell compartment of the bird is derived. Chicken Ig gene rearrangement is restricted to the developing embryo and generates sIg receptors of very limited diversity (15, 16). The specificity of these receptors, however, is not important for the early stages of bursal colonization, since we have recently demonstrated (17) that the colonization of bursal follicles can be supported by the cell surface expression of a truncated Ig \( \mu \) heavy chain (T\( \mu \)). The T\( \mu \) molecule lacks the VDJ\( \mu \) and C\( \mu \)1 domains and, as a consequence, neither binds Ig light chain nor requires the presence of light chains for its surface expression on B cell precursors.

Those B cell precursors that productively colonize bursal follicles as a consequence of either endogenous sIgM expression or expression of the T\( \mu \) protein undergo rapid cell division during embryonic life (18), and the number of B-lineage cells within the bursa doubles every day for the last 10 days of embryonic life (19). Several experimental approaches have demonstrated that the B cells in each follicle are derived from a limited number (two to five) of sIg\( ^{+} \) precursors and, further, that there is no traffic of B-lineage cells from one follicle to another (10, 20, 21). As a consequence, the increase in bursal cellularity observed after about embryonic day 15, when most immigration of B cell precursors to the bursa has ended, represents the consequences of B cell division within the bursa. Ig diversity is generated among this population of rapidly dividing sIg\( ^{+} \) bursal cells (14, 15, 22). In chickens (1, 3, 14, 15), as in some other mammalian species (23, 24), Ab diversity is generated by a process of gene conversion. Sequences derived from upstream pseudo-V genes replace homologous sequences in the unique V\( _{\mu} \)1 and V\( _{\lambda} \)1 genes following their rearrangement to DJ\( _{\mu} \) and J\( _{\lambda} \), respectively. We have demonstrated that although B cell precursors expressing T\( \mu \) do not express endogenous sIgM, some contain endogenous gene rearrangements (17). Analysis of V\( J_{\lambda} \) sequences from neonatal bursal cells expressing T\( \mu \) has demonstrated that they have undergone as much diversification by gene conversion as V\( J_{\lambda} \) sequences in cells expressing endogenous sIg receptors (25). Consequently, T\( \mu \) expression by B cell precursors is sufficient to support the stages of B cell development that occur in the avian embryo, namely follicular colonization with the onset of B cell proliferation and the induction of Ig diversification by gene conversion.

The physiology of the bursa undergoes a series of changes around the time of hatching. The rate of bursal cell growth, as judged by the number of B-lineage cells within the bursa, slows (19). Rather than doubling in cell number every day during embryonic life, cell numbers double about every 7 days. Some cells...
migrate from the epithelial bud back across the basement membrane to form an outer cortex of cells surrounding a central medulla, and the basement membrane develops into a complex cortico-medullary junction of interdigitating cells (26). As the cortico-medullary distribution of lymphocytes develops in the posthatching period, cortical cells continue to undergo rapid cell division. In contrast medullary lymphocytes have markedly reduced levels of cell division (18, 27, 28).

Based on calculations of the rates of bursal cell division and emigration of bursal cells to the periphery (29, 30), it has been estimated that only about 5% of the cells produced in the juvenile bursa emigrate to the periphery. The great majority of bursal cells die in situ by apoptosis (31, 32). Although bursal cells that lose the expression of sIg die in situ (32), it is unclear whether the loss of sIg expression is the trigger responsible for all bursal cell death. In this regard, gene conversion in the chick embryo efficiently maintains the productive reading frame of the IgVL gene even under circumstances where bursal cell viability is maintained by expression of the Tµp protein and not by endogenous sIg expression (25).

The bursa is a gut-associated lymphoid tissue. Exogenous and gut-derived Ags are actively transported across the bursal epithelium into the lymphoid follicles of the bursa (33–36). Therefore, the antigenic environment of B cells in the bursa after hatching differs from that of bursal cells developing in the embryo. At present, however, the consequences of bursal cell exposure to Ag remain unclear. Ligation of the bursal duct before hatching blocks the transport of gut-derived Ags and/or mitogens into the bursa and results in reduced proliferation of bursal cells after hatching (27). In addition, introduction of Ag into bursal follicles has been shown to result in increased Ab response in the periphery following subsequent systemic challenge (37). Two distinct populations of peripheral B cells have been identified as emigrating from the bursa (30, 38), leading to speculations that the maturation and/or emigration of at least some bursal cells might require a cognate interaction with Ag.

In this paper we have addressed the question of whether discrete physiological mechanisms regulate the growth and development of B cell precursors in the bursa before and after hatching. We show that while expression of the Tµp protein is sufficient to support normal bursal cell development in the embryo, cells expressing Tµp are selectively eliminated from the bursa after hatching. Elimination of Tµ⁺ bursal cells occurs both in follicles that also contain cells expressing endogenous sIgM receptors and in follicles containing exclusively Tµ⁺ cells. We can conclude, therefore, that the elimination of Tµ⁺ cells occurs independently of competition for intrafollicular space within the bursa. These results provide the first direct evidence that the Ag-binding Fab domains of the sIgM molecule play a critical role in avian B cell development.

**Materials and Methods**

**Generation of RCAS-Tµ-infected chicks**

RCAS-Tµ- or control RCAS virus-infected chicks were generated as described previously (17). On day 3 of incubation, 1 million RCAS- or RCAS-Tµ-transfected line 0 embryo fibroblasts were injected in ovo into SC line (Hyline International, Dallas Center, IA) embryos.

**Cell suspensions and flow cytometry**

Bursal cells and thymocytes were prepared and stained with 11C6 (anti-chicken Ig light chain), Hy18 (anti-chicken µ), EP96 (anti-CD4), or EP72 (anti-CD8α) as described previously (9, 17, 22, 39). Binding of primary Abs was detected using FITC-, R-PE-, or biotin-conjugated goat anti-mouse Ig isotype-specific secondary reagents (Southern Biotechnology Associates). Viable cells were analyzed or sorted on a FACS Vantage (Becton Dickinson Canada, Mississauga, Canada) by gating on forward scatter and side scatter. Viable cells were sorted with additional gating for the exclusion of cells staining with propidium iodide (0.5 µg/ml).

For determination of cellular DNA content, stained bursal cells were FACs sorted to >98% purity, pelleted, and resuspended for 10 min at 37°C in Vindelev’s solution (3.4 mM Tris, 7.5 × 10⁻³ M propidium iodide, 0.1% (v/v) Nonidet P-40, and 700 µL RNase A in 10 mM NaCl, pH 7.6) as described previously (40). The DNA content of the resulting nuclei was then determined on a Becton Dickinson FACScan.

**Immunohistochemistry**

Bursae from hatched chicks were extracted and frozen in crushed dry ice for 2 min. The frozen tissues were then embedded in OCT and stored at −70°C. Eight-micron sections were cut in a refrigerated microtome at −17°C and mounted on SuperFrost Plus slides (Fisher Scientific, Nepean, Canada). The sections were dried overnight at room temperature before use. Slides were rehydrated in 50 mM Tris in 0.15 M NaCl, pH 7.4 (TBS), for 10 min, then washed three times, each for 10 min, with 0.09% (v/v) Tween 20 in TBS followed by two washes in TBS. Tissues were fixed in freshly prepared 4% paraformaldehyde in 70 mM NaHPO₄, pH 7.0, for 10 min and washed again twice for 5 min each time in TBS. Endogenous peroxidase activity was quenched using a 3% peroxide/methanol solution (1/4) for 30 min. This was followed by two more washes in TBS for 5 min each. The slides were then blocked in TBS with 2.5% FCS for at least 30 min in a humidified box at room temperature.

Sections were stained with the following mouse mAbs: anti-chicken µ, κ, and λ, generated as described previously (22) at 30 µg/ml, anti-chicken light chain 11C6 (22) at 2.5 µg/ml, or a combination of 21-1A4 and FUS11G2 (anti-Chb6.1 and anti-Chb6.2, respectively) (41), each at 20 µg/ml.

Primary Abs were diluted in blocking buffer, and slides were incubated for 2 h at room temperature in a humidified box, after which they were washed three times in TBS. Binding of primary Abs was detected with biotin-conjugated goat anti-mouse Ig containing nonmodified streptavidin-spectral red (Southern Biotechnology Associates) or a biotinylated goat anti-mouse Ig containing nonmodified streptavidin (Southern Biotechnology Associates). Viable cells occurring independently of competition for intramedullary space within the bursa. These results provide the first direct evidence that the Ag-binding Fab domains of the sIgM molecule play a critical role in avian B cell development.
signals are provided by the 3′ long terminal repeat of the RCAS virus (42, 43).

We have previously shown that B cells expressing a truncated form of slgμ (Tμ) develop in the lymphoid follicles of the avian embryonic day 21 bursa from RCAS-Tμ-infected chicks stained for the expression of IgL (A and D), the ChB6 pan-B cell marker (B and E), and μ (C and F). This bursa contained 18.5% μ−L− cells as judged by flow cytometry. A follicle containing exclusively μ−L− cells is indicated by the arrow in A. Follicles appearing to contain exclusively μ−L− cells are indicated (+) in A and D. Follicle containing a mixture of μ−L− and μ+L+ cells is indicated by arrowheads in A and D. Bar = 100 μm.

FIGURE 1. Bursal follicles in RCAS-Tμ-infected chicks can contain exclusively μ−L− cells. Frozen sections of embryonic day 21 bursa from RCAS-Tμ-infected chicks stained for the expression of IgL (A and D), the ChB6 pan-B cell marker (B and E), and μ (C and F). This bursa contained 18.5% μ−L− cells as judged by flow cytometry. A follicle containing exclusively μ−L− cells is indicated by the arrow in A. Follicles appearing to contain exclusively μ−L− cells are indicated (+) in A and D. Follicle containing a mixture of μ−L− and μ+L+ cells is indicated by arrowheads in A and D. Bar = 100 μm.

Because bursal follicles in the embryonic bursa are oligoclonal, we would expect the majority of bursal follicles in RCAS-Tμ-infected chicks to contain both μ−L− cells expressing endogenous slgM and μ+L+ cells expressing Tμ under conditions where the bursa overall contains a mixture of both cell types. Indeed, bursal follicles of RCAS-Tμ-infected chicks frequently stain heterogeneously with anti-IgL Abs (Fig. 1, D–F), indicating the presence of oligoclonal bursal follicles containing both μ−L− and μ+L+ bursal cells.

The frequency of follicles containing exclusively μ−L− cells is low (<3% of follicles) relative to the total proportion of μ−L− cells in bursal cell suspensions of neonatal bursae (typically 10–25%), indicating that the majority of μ−L− bursal cells proliferate in mixed follicles that also contain μ+L+ cells. This is consistent with the oligoclonality of bursal follicles and suggests that the efficiency with which μ−L− precursors colonize bursal follicles is equivalent to that seen with μ+L+ precursors expressing endogenous receptors.

We have previously demonstrated that the proportion of μ−L− cells in the bursa of neonatal chicks infected as day 3 embryos with RCAS-Tμ is about 20% (17). Surprisingly, flow cytometric analysis of bursal cell suspensions revealed that the proportion of μ−L− bursal cells in RCAS-Tμ-infected chicks decreased rapidly in the first 2 wk after hatching (Fig. 2). By 2 wk of age, most chicks contained <1% μ−L− cells and four of nine contained <0.3% μ−L− bursal cells. RCAS-based viruses infect both non-B as well as B-lineage cells (41), and the Tμ protein is expressed on the surface of non-B cells (data not shown). The proportion of CD4- and/or CD8-expressing thymocytes that coexpressed the Tμ protein was typically...
in the range of 0.5–2.0% (Fig. 2). Crucially, however, the proportion of $\text{T}^\mu_1$ thymocytes did not change over the first 2 wk after hatching. In particular, the four 14-day-old chicks that contained $0.3\%$ $\text{T}^\mu_1L^2$ cells in the bursa contained significant numbers of $\text{T}^\mu_1$ thymocytes. Therefore, the elimination of $\text{T}^\mu_1L^2$ cells during this time is specific for events occurring within the bursa as opposed to a generalized loss of cells expressing the retrovirally transfected $\text{T}^\mu$ protein.

At the time of hatching, the rate of cell division among FACS-sorted $\text{T}^\mu_1L^2$ bursal cells is indistinguishable from that seen with $\text{T}^\mu_1L^1$ cells and exhibits the high rate of proliferation characteristic of the rapid expansion of B cells in the bursa of neonatal chicks (Fig. 3, $D$ and $E$), confirming previous results (17–19). The growth rate of normal bursal cells declines after hatching, and this is reflected in the proportion of $\text{T}^\mu_1L^1$ cells in S, G2, and M phases of the cell cycle on day 5 (Fig. 3 $F$). However, $\text{T}^\mu_1L^2$ bursal cells of day 5 chicks show a dramatic reduction in proliferation compared with $\text{T}^\mu_1L^1$ bursal cells from the same chicks (Fig. 3 $G$). Therefore, the rapid disappearance of $\text{T}^\mu_1L^2$ cells can be accounted for at least in part by a reduction in their rate of proliferation.

In addition to a decrease in the proportion of cells in S, G2, and M phases of the cell cycle, sorted $\text{T}^\mu_1L^2$ cells from RCAS-$\text{T}^\mu$-infected day 5 chicks also contained a significant frequency (<10%) of cells containing $2N$ DNA compared with $\text{T}^\mu_1L^1$ cells (<2%) from the same chicks (Fig. 3, $H$ and $J$). Hypodiploid DNA content is a characteristic of bursal cells undergoing apoptotic cell death (31, 32), suggesting that the loss of $\text{T}^\mu_1L^2$ cells after hatching was not simply a consequence of reduced rates of cell division resulting in the dilution of these cells, but rather was a consequence of their active elimination by apoptosis.

Under normal circumstances, apoptotic cells are rapidly phagocytosed by tissue macrophages (44, 45). Assessment of apoptotic...
The frequency and distribution of apoptotic cells in situ within the bursa, because the analysis was restricted to cells of lymphoid size, and macrophages containing phagocytosed apoptotic remnants would be excluded from the analysis. The frequency and distribution of apoptotic cells in situ within the bursa were therefore determined by TUNEL staining of paraffin-embedded bursal sections (Fig. 4), followed by counting the number of apoptotic cells per bursal follicle section (Fig. 5).

On embryonic day 21, within 12–24 h of hatching, there was little difference in the frequency or distribution of apoptotic cells when comparing sections from RCAS-Tm- or RCAS-infected chicks (Figs. 4, A and B, and 5A). The frequency of apoptotic cells in RCAS-infected chicks did not differ from that in uninfected control chicks (data not shown). Most follicles contained small numbers of apoptotic cells, and these cells were typically distributed evenly in both RCAS-Tm and RCAS bursae. Only in one bursa, which contained nearly 80% of μ-L− cells, was there a significant increase in the proportion of follicles containing >20 apoptotic cells/follicle section (8% compared with 3% in the control bursa).

Within 2 days after hatching, however, there was a marked difference in the frequency and distribution of apoptotic cells when comparing bursal sections from RCAS-Tm- and RCAS-infected chicks (Figs. 4, C and D, and 5B). The frequency of apoptotic cells in RCAS-infected bursae was increased after hatching compared with that in embryonic day 21 bursae. However, in sections from RCAS-Tm-infected chicks the average number of apoptotic cells per follicle section was increased compared with that in age-matched RCAS control sections, and there was a striking increase in the frequency of follicles containing a high number of apoptotic nuclei per follicle. In particular, the frequency of follicles that contained 20 or more apoptotic nuclei increased to >35% of all follicles in the section compared with 10% of follicles in control sections from RCAS-infected chicks. Conversely, there was a marked reduction in the frequency of bursal follicles with zero to three noticeable apoptotic events per follicle in RCAS-Tm-derived sections compared with that in RCAS control sections.

There were also differences in the distribution of apoptotic cells within bursal follicles of RCAS-Tm-infected chicks after hatching compared with that in control bursae. Apoptotic cells in control bursal sections were typically distributed evenly as single cells within follicles, with <5% of follicles containing >10 phagocytosed nuclei/follicle on day 2 after hatching (Figs. 4E and 5C). In contrast, the follicles from RCAS-Tm-infected chicks contained a significant proportion of apoptotic cells within clusters, many of which had the appearance of tissue macrophages that had ingested several apoptotic cells (Fig. 4F). The frequency of follicles from RCAS-Tm-infected chicks containing >10 phagocytosed nuclei on day 2 after hatching was about 20% of follicles.

The distribution of apoptosis in bursal follicles from RCAS-Tm-infected chicks was not even. Individual sections contained follicles with little apoptosis as well as follicles with considerable apoptosis (Figs. 4D and 5B). For example, 35% of follicle sections from Tg57 contained 20 or more apoptotic cells, while 10% of follicles contained three or fewer apoptotic cells. We therefore determined whether follicles from RCAS-Tm-infected chicks that contained exclusively μ−L− cells contained elevated levels of apoptotic cells or, alternatively, whether these follicles exhibited reduced levels of apoptosis. Serial frozen sections of bursal tissue from RCAS-Tm-infected chicks were assayed for apoptotic cells by TUNEL and for IgL and either Igμ or ChB6 to identify follicles containing exclusively μ−L− cells. On days 2–3 of age most follicles containing exclusively μ−L− cells contained high levels of apoptotic cells (Fig. 6). Not surprisingly, given that the majority of μ−L− cells develop in mixed follicles that also contain μ+L− cells, some highly apoptotic follicles also stained heterogeneously for the expression of IgL, demonstrating that they do indeed contain μ+L− cells expressing endogenous receptors (Fig. 6, G and H). Nonetheless, the high levels of apoptosis in follicles containing exclusively μ+L− cells demonstrates that elimination of μ+L− cells by apoptosis is not a consequence of competition with cells expressing endogenous sIgM for physiological space within a follicle.

**Discussion**

During normal embryogenesis the avian bursa of Fabricius is colonized by B cell precursors that have productively rearranged Ig genes. These precursors proliferate in lymphoid follicles termed epithelial buds. The mechanism by which local tissue reorganization results in the formation of lymphoid follicles following B cell
precursor migration across the bursal basement membrane is currently unclear. In the mouse spleen, the induction of B cell follicles containing mature follicular dendritic cells requires the expression of lymphotoxin-α on the surface of B cells themselves, suggesting that B cells play an integral role in modeling their own developmental environment (46, 47). We have previously demonstrated that expression of a truncated μ heavy chain on the surface of B cell precursors is sufficient to allow their development within lymphoid follicles of the embryo bursa. Cells expressing Tμ do not express endogenous slg and therefore do not express V(D)J-encoded determinants of the slg receptor. Since we show here that bursal follicles can contain exclusively μ+L− cells expressing the Tμ receptor, an interaction between the prediversified receptor and ligand(s) expressed on the bursal stroma is not required for the formation of bursal follicles.

On the average, μ+L− bursal cells in neonatal RCAS-Tμ-infected chicks accounted for 15–20% of total bursal B cells. However, the frequency of bursal follicles that uniquely contained μ+L− precursors was typically about 2%, indicating that the majority of μ+L− bursal cells develop in follicles containing both μ−L+ and μ+L− cells. This is consistent with several reports demonstrating that a limited number of B cell precursors (between two and five) productively colonize each bursal follicle during embryogenesis and that there is no interfollicular trafficking of lymphoid cells within the bursa (10, 21, 22). Indeed in the case of a bursa containing 25% μ+L− cells, in which each bursal follicle is colonized by three precursors, one would expect about 1.5% of follicles to have been colonized by three μ+L− precursors. Our results therefore are consistent with the oligoclonal colonization of bursal follicles by two or three precursors and furthermore demonstrate that the efficiency of bursal follicle colonization by μ−L+ precursors is equivalent to that seen with μ+L− precursors.

μ+L+ and μ−L+ cells from neonatal chicks proliferate at the same rate (Fig. 3, D and E) typical of the rapid expansion of bursal cells during embryonic development (18, 19). Taken together with the observation that most μ+L− bursal cells develop in oligoclonal follicles that also contain μ+L+ cells, these results indicate that μ+L− precursors are at no intrafollicular competitive disadvantage with respect to their proliferation during embryogenesis in the presence of μ+L+ precursors. In contrast, anergic murine B cells persist in the periphery only in the absence of competition from nonanergic B cells. In the presence of competing B cells, anergic B cells are excluded from follicular niches and are quickly eliminated (48, 49). The observation that μ+L− cells in the bursa develop initially at normal rates despite the presence of competitor B cells expressing endogenous receptors supports our conclusion that these cells are undergoing a normal program of development during embryogenesis. This conclusion is further strengthened by the demonstration that at the time of hatching the small proportion of μ+L− cells in the bursa of RCAS-Tμ-infected chicks that have rearranged the endogenous Vβ loci (17) has undergone as much IgVβ diversification by gene conversion as their μ+L+ counterparts (25).

Surface expression of either endogenous slgM or Tμ is required for B cell expansion in the embryo bursa. Because the chicken slgM complex includes homologues to CD79a (Igα) and CD79b (Igβ), and both endogenous slg and Tμ (50, 51) can transduce signals across the plasma membrane, constitutive basal signaling through the slg complex may be sufficient to support B cell development in the embryo. This is analogous to the requirement for continuous slg expression in the murine spleen, where B cell survival depends on constitutive basal signaling through slg (52). The disappearance of cells expressing Tμ in the posthatching period, however, demonstrates that such basal signaling is not sufficient to support bursal cell development after hatching.

μ+L− cells after hatching drop out of cell division, as demonstrated by reduced levels of cells in S, G2, and M phases of the cell cycle, and undergo apoptotic cell death. We have previously demonstrated that cell death in bursal cells from juvenile chickens is preceded by the loss of slg (31). It was argued that the loss of slg could be induced in part by nonproductive gene conversion events leading to out-of-frame Vβ gene segments. However, μ+L− bursal cells from RCAS-Tμ-infected chicks express the Tμ receptor independent of the status of the endogenous Ig genes. Therefore,
failed gene conversion events cannot account for the loss of $\mu^+L^-$ bursal cells after hatching.

Several lines of evidence suggest that the cellularity of the bursa is closely regulated by homeostatic mechanisms that are currently undefined but probably reflect competition for physiological space among developing bursal cells. Thus, in IgsM-1$^+$/IgsM-1$^-$ allotype heterozygous chicks, deletion of bursal cells expressing the slgM-1a allotype results in a compensatory expansion of cells expressing the alternative slgM-1b allotype (53). Similarly, bursal follicles experimentally colonized by single precursor cells rapidly attain the same cellularity as follicles naturally colonized by several precursor cells (20). It is currently unclear whether the elimination of $\mu^+L^-$ cells requires the presence of competing $\mu^+L^+$ B cells within the bursa, because the RCAS-T$\mu$ chicks described here contain bursal cells expressing endogenous slgM receptors. Nonetheless, the elimination of $\mu^+L^-$ cells from follicles that do not contain $\mu^+L^-$ demonstrates that there is clearly no requirement for intrafollicular competition from $\mu^+L^+$ bursal cells. If interfollicular competition is required for the elimination of $\mu^+L^-$ cells, it must operate by soluble intermediates, because there is no traffic of B cells from one follicle to another.

The bursa as well as the lymphoid cells within it undergo a series of physiological changes that are initiated in the perinatal period. During embryonic development, lymphoid cells proliferate in epithelial buds, resulting in an exponential increase in the number of slg$^-$ cells within the bursa, such that B cell numbers double every day. After hatching, however, the rate at which the number of B cells in the bursa increases slows dramatically, such that bursal B cell numbers double every 7 days (19). This is consistent with the reduced number of cells in S, G2, and M phases of the cell cycle in $\mu^+L^+$ bursal cells on day 5 (22%; Fig. 3F) compared with that on the day of hatching (46%; Fig. 3D). In addition, starting around the time of hatching, B cells in the epithelial buds segregate to form an outer cortex of cells surrounding the inner medulla. After this occurs, most bursal cell proliferation occurs in the cortex of bursal follicles, the medulla contains largely nondividing cells (18, 27, 28). The factors determining which B cells within the epithelial bud migrate to form the cortex of rapidly dividing cells within the bursal follicle are currently unknown. The results presented here suggest, however, that expression of T$\mu$ is not sufficient to support this development.

The frequency of apoptotic cells in the normal bursa increases after hatching. Thus, in control sections the average number of apoptotic cells per follicle increased between embryonic day 21 (Figs. 4A and 5A) and day 2 after hatching (Figs. 4C and 5B). The mechanism by which the frequency of apoptosis in the bursa increases after hatching is unclear, although glucocorticoid concentrations in the embryo increase around the time of hatching (54), and injection of glucocorticoids into the hatch chick induces bursal cell death (55).

There are several lines of evidence that would be consistent with a role for Ags and/or mitogens in promoting the development of bursal cells after hatching. Gut-derived Ags are transported by pinocytosis from the bursal lumen to the epithelial bud or medulla of bursal follicles under conditions where they can physically interact with developing bursal lymphoid cells (33–36). Ligation of the bursal duct blocks the traffic of gut-derived Ags into the lymphoid compartment of the bursa. Although this treatment does not impede either the rate of B cell expansion in embryo epithelial buds (27) or the rate of Ab diversification by gene conversion during embryonic development (56), the rate of B cell growth in the posthatching bursa is reduced. A similar situation occurs when bursal epithelia are grafted into the abdominal wall (12). Such epithelia become colonized by bursal precursors and develop normally during embryonic life under conditions where they are isolated from gut-derived Ags. After hatching, however, these ectopic bursae involute.

One possible interpretation of these results is that bursal cell development after hatching is driven by gut-derived mitogen(s). This is difficult to reconcile with the failure of T$\mu^-$ bursal cells to develop normally after hatching, because we have been unable to demonstrate any difference in phenotype between T$\mu^-$ cells and cells expressing endogenously encoded slgM, using a large range of mAbs that recognize bursal cell surface Ags (17, 57). In addition, there is no a priori reason why expression of T$\mu$ should inhibit the expression of a mitogen receptor or block the function of that receptor.

The T$\mu$ protein lacks the VDJ$\mu$ and C$\mu$1 domains. As a consequence it cannot bind light chain and does not require association with light chain for surface expression; indeed, the great majority of T$\mu^+$ cells do not contain a functionally rearranged light chain gene (17). Consequently, the major difference between T$\mu^-$ cells and cells expressing endogenously encoded slgM is that the latter can bind Ag by means of the V(DJ)-encoded $\mu$ and light chain V domains. The results presented here demonstrate that slg molecules that lack Ag-binding capacity fail to promote the development of bursal cells after hatching. The repertoire of developing bursal cells has been extensively diversified by gene conversion by the time of hatching (13–16), so posthatching bursal development cannot be driven by a small set of B cell specificities. Posthatching bursal cell development may therefore be driven by specific interactions between the slg receptor and multiple gut-derived Ags presented within the bursal follicle. The presence of cells in the bursa expressing immune complexes on their surface would be consistent with this view (58). Alternatively, V(DJ)-encoded determinants in the bursa may be ligated by a B cell superantigen, as has been proposed in other species (59, 60). The requirement for slg ligation at a time when endogenous glucocorticoids are elevated would suggest the possibility that bursal cells become susceptible to glucocorticoid-induced cell death in the perinatal period unless Ag or superantigen induces slg ligation. A balance between glucocorticoid-induced death and Ag receptor ligation has been proposed to regulate the selection of T cells in the developing thymus (61, 62).

In conclusion, we have demonstrated that during embryonic development, bursal follicles in RCAS-T$\mu$-infected chicks can be colonized exclusively by $\mu^+L^-$ cells lacking endogenous slgM receptors. We show that $\mu^+L^-$ cells are rapidly lost from the bursa after hatching as a consequence of decreased cell division rates and by the selective increase in apoptosis of $\mu^+L^-$ cells compared with that of $\mu^+L^-$ bursal cells expressing endogenous receptors. The high level of apoptosis in bursal follicles containing exclusively $\mu^+L^-$ cells after hatching demonstrates that apoptotic elimination of such cells occurs independently of intrafollicular competition from bursal cells expressing endogenous receptors.

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