Loss of Surface Immunoglobulin Expression Precedes B Cell Death by Apoptosis in the Bursa of Fabricius

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Summary

The vast majority of lymphocytes generated daily in the chicken bursa of Fabricius do not emigrate to the periphery but die in situ. Apoptotic cells in the bursa can be readily detected by the presence of fragmented DNA and by the large numbers of condensed cellular nuclei observed by electron microscopy. Consequently, most newly generated lymphocytes die by programmed cell death. We show that bursal cells divide rapidly and apoptotic cells are derived from rapidly dividing precursors. Analysis of the phenotype of bursal cells undergoing apoptosis demonstrated that cell death does not occur in the most mature bursal cell population and is therefore not random. High levels of surface Ig are expressed on bursal cells entering S phase of the cell cycle. In contrast, bursal cells in the early stages of apoptosis in vivo express very low to undetectable levels of surface Ig but were unequivocally confirmed as being of the B lineage by polymerase chain reaction (PCR) detection of rearranged Ig genes. Bursal cells induced to undergo apoptosis in vitro express high levels of surface Ig demonstrating that induction of apoptosis does not in itself induce a loss of surface Ig expression. Consequently, loss of surface Ig expression precedes bursal cell death by apoptosis in vivo, suggesting that maintenance of a threshold level of surface Ig may be a requirement for the continued progression of chicken B lymphocyte development in the bursa.

The bursa of Fabricius is the primary lymphoid organ responsible for the establishment and maintenance of the B cell compartment in avian species. It contains actively dividing cells expressing surface Ig from a few days before hatching until its involution at sexual maturity at about 6 mo of age. The bursa appears not to be required for the induction of Ig gene rearrangement and surface Ig expression among B cell precursors (1–3). There is considerable evidence, however, that the normal progression of B cell development and extensive diversification of the B cell repertoire by Ig gene conversion require the presence of the bursa (4, 5).

There is extensive cell division in the bursa, particularly in the first few weeks of life, during which time the bursa is increasing in cellularity as well as producing B cells that emigrate to the periphery (6). Direct measurements of the rate of emigration of bursal lymphocytes to the periphery has shown that only 5% of the cells generated daily emigrate to the periphery (7). The prediction that most of the newly generated bursal lymphocytes die in situ was confirmed by the demonstration of bursal cells undergoing programmed cell death in situ (8).

Cell death is frequently observed among lymphoid precursor cells and occurs by apoptosis, an active, protein synthesis dependent process, which results in cleavage of genomic DNA into multiples of ~180 bp. Additional phenotypic changes occurring later in the process include nuclear condensation and cellular membrane blebbing (reviewed in 9, 10). Apoptosis has been observed among B lineage cells developing in murine bone marrow (10a) and it is possible that this apoptosis is a consequence of the failure to produce functional cell surface Ig due to the accumulation of nonproductive gene rearrangement events. Ig gene rearrangement among chicken B cell precursors, however, is restricted to the developing embryo (11). Consequently, apoptotic cell death in the post-hatching bursa cannot be a result of nonproductive gene rearrangement events. Among developing T cells, apoptosis has been associated with the intrathymic processes of negative selection and the failure of thymocytes to be positively selected (12).

Recent evidence has demonstrated functional (13) and phenotypic (Paramithiotis, E., and M. J. H. Ratcliffe, manuscript in preparation) heterogeneity among the 5% of bursal cells generated daily that emigrate to the periphery. This suggests the potential for (a) selective process(es), of an as yet undefined nature, that determine which bursal cells will emigrate to the periphery and which will undergo apoptotic cell death in situ. Here we demonstrate that loss of B lymphocyte surface Ig expression precedes death by apoptosis in the bursa. Consequently the maintenance of high levels of surface Ig expression is required for the continued progression

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of bursal cell development, whereas those cells that have lost the expression of surface Ig are functionally and physically eliminated from the B cell lineage by apoptotic cell death.

Materials and Methods

Cell Suspensions and DNA Samples. Bursal cell suspensions were prepared from 3–5-wk-old SC Hyline chickens (Hyline International, Dallas Center, IA) by passing the tissues through a wire mesh. The tissues were washed in ice cold Hank's balanced salt solution without Ca^{2+} or Mg^{2+} as described (8). Bursal lymphocyte suspensions were cultured at a concentration of 0.5×10^6 cells/ml in Iscove's modified Dulbecco's medium with 5% FCS and 2% normal chicken serum (GIBCO BRL, Burlington, Ont., Canada) as described elsewhere (3).

Low molecular weight DNA was purified from 10^8 bursal cells and PBL, and was analyzed by electrophoresis and ethidium bromide staining as described (8).

Bursal tissue samples were prepared for electron microscopy also as described elsewhere (14).

In vivo bromodeoxyuridine (BrdUrd;[1] Sigma Chemical Co., St. Louis, MO) labeling (50 mg/kg i.p.) was done as described elsewhere (15). Vincristine sulfate (Sigma Chemical Co.) was injected intravenously at 0.5 mg/kg bodyweight 4 h before harvesting bursal tissue (16).

Antibodies and Flow Cytometry. mAbs 4C3.B10 (IgM anti-Ig light chain; reference 15), 6E4 (IgG1 anti-Ig light chain; reference 17), and 11A9 (IgM anti-chL12; reference 18) were affinity purified from hybridoma supernatants using an anti-mouse κ (187.1; reference 19) Sepharose column and used at 10 μg/ml for staining. mAbs EP25 (IgG1 anti-MHC class II), Bromo-2 (IgG1 anti-BrdUrd; reference 15), and a mixture of FU5.11G2 (IgG1 anti-chB6.2; reference 20) and 21-1A4 (IgG1 anti-chB6.1; reference 20) were used as culture supernatants.

For two-color analysis of cellular DNA content and cell surface antigen expression, cells were fixed with 70% ice cold ethanol for 30 min, and then washed three times with Dulbecco's PBS, incubated with Dulbecco's PBS with 2.5% FCS for 15 min at room temperature, and then incubated with the primary mAb for 15 min at room temperature. The cells were subsequently washed twice with Dulbecco's PBS/FCS before being resuspended in 50 μl of the appropriate goat anti-mouse isotype FITC conjugate (Southern Biotechnology Associates, Birmingham, AL) for 15 min. The cells were then washed as before, resuspended in Dulbecco's PBS/FCS, 50 μg/ml RNase (Sigma Chemical Co.) and 0.03 mg/ml propidium iodide (PI; Sigma Chemical Co.) as described (13), and incubated for 5 min at 37°C before analysis. Cell suspensions in Ca^{2+} Mg^{2+} free Hanks were processed for detection of BrdUrd incorporation as described (15).

Two-color analysis of BrdUrd incorporation and DNA content was done by staining first for detection of BrdUrd as described elsewhere (13) followed by incubation with PI (1 μg/ml) as above. Samples were analyzed on a FACScan® (Becton Dickinson Canada Inc., Missisauga, Ont., Canada) by gating on forward and side scatter. Flow cytometric cell sorting was performed on cells stained after fixation with anti-chB6 and PI using a FACStar plus®. Cells were sorted by gating on chB6 expression and DNA content.

PCR Analysis of Ig Gene Rearrangement. PCR was used to detect rearranged (VJ) Ig V_L1 and rearranged (VDJ) Ig V_H1 genes as described elsewhere (21). The oligonucleotide primer combinations (Sheldon Biolabs, McGill University, Montréal, Québec, Canada) were: VL5′ (5′-TGTCCCATGGCTGCGCGGGCAGGGCTGT-3′) with JL3′ (5′-AGAAAGATCTAGACGAGGTCAGCGACTC-3′) to amplify rearranged light chain VJ, and VH5′ (5′-GTGGCCGCGGCTCCGTCAGCGCTCTCTG-3′) with JH3′ (5′-GGTTGATCACTCACCGGAGGAGACGATG-3′) to amplify rearranged heavy chain VDJ. PCR reactions consisted of 50 ng DNA template, 25 pmol of each primer, 1.25 mM dNTPs, 10% dimethylsulphoxide, 67 mM Tris, 16.6 mM NH_4SO_4, 10 mM 2-mercaptoethanol, and 5–10 mM $MgCl_2$. PCR reactions were performed with 2 U of Taq DNA polymerase (Perkin Elmer Cetus, Etobicoke, Ont., Canada) in a thermal cycler (Hypercell Biological, Chalk River, Ont., Canada) for 30 cycles, followed by a 20-min extension reaction at 72°C. Each cycle for the amplification of rearranged heavy and light chain consisted of 93°C for 1.5 min, 60°C for 1.5 min, followed by 72°C for 1 min. PCR-amplified products were electrophoresed and visualized on 2% agarose gels containing 0.5 mg/ml of ethidium bromide.

Results

Cell Death Is Not Randomly Distributed among Bursal Lymphocyte Subpopulations. There is extensive cell division in the bursa of juvenile chickens, yet only a small minority, estimated at about 5% of the lymphocytes produced daily (7), emigrate to the periphery. The majority of the bursal cells produced daily, therefore, are expected to die in situ. Fig. 1 *a* confirms the presence of significant amounts of fragmented DNA in bursal lymphocytes but not in PBL. This corresponds to ~7% of total bursal DNA (reference 2 and data not shown). Fig. 1 *b* demonstrates the presence of condensed nuclei, characteristic of the late stage of apoptotic cell death. The condensed nuclei are largely found within phagocytic cells (e.g., Fig. 1 *b*) located in both the cortical and medullary regions of bursal follicles (data not shown).

Flow cytometric analysis of bursal cell DNA content revealed, in addition to extensive cell division, a population of about 5% of bursal cells containing less than 2 N DNA (Fig. 1 *c*). These cells were viable as judged by trypan blue exclusion. Cells containing <2 N DNA and high levels of chB6 (Bu-1; a pan-B cell marker found on all bursal lymphocytes) were purified by flow cytometric cell sorting and examined by electron microscopy. All purified cells were in the early stages of apoptotic cell death (Fig. 1 *d*). Consequently flow cytometric detection of cells with <2 N DNA provides a reliable indicator of cells undergoing apoptosis in vivo. It should be emphasized that the flow cytometric assay for cells containing <2 N DNA detects only those bursal cells that have begun to die by apoptosis and have not yet undergone phagocytosis.

We have previously demonstrated by the incorporation of BrdUrd that the great majority of bursal lymphocytes are rapidly dividing (15). To determine the relationship between bursal cell division and cell death, we injected BrdUrd in vivo and measured the rate of appearance of BrdUrd-labeled bursal lymphocytes containing <2 N DNA (Fig. 2). BrdUrd was rapidly incorporated into both the viable and apoptotic bursal populations, the latter with a delay of about 4 h compared

[1] *Abbreviations used in this paper:* BrdUrd, bromodeoxyuridine; PI, propidium iodide.

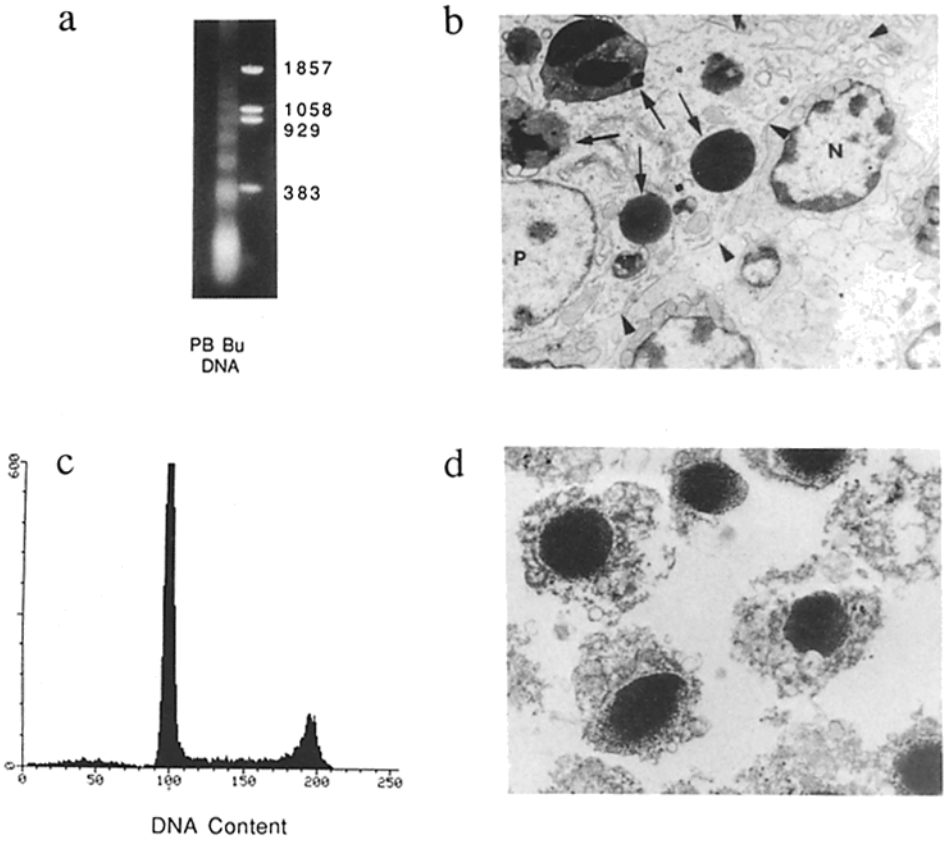

Figure 1. Apoptosis among bursal lymphocytes. (*a*) Low molecular weight DNA from 10^8 PBL (PB) or bursal lymphocytes (Bu) as determined by electrophoresis. Numbers on the right refer to size markers (in bp). (*b* and *d*) Electron micrographs of bursal sections (*b*) and cells containing <2 N DNA purified by cell sorting (*d*). Condensed nuclei in (*b*) are marked with *arrows*. A representative normal lymphocyte nucleus is marked with *N*. The membrane of a phagocytic cell is marked with *arrowheads* and its nucleus with *P*. Original magnification for (*b*) and (*d*) is at 5,000. (*c*) Flow cytometric analysis DNA content in ex vivo bursal cells. 10,000 events are displayed gated on forward and side scatter.

with the rate of accumulation into cells with ≥2 N DNA. Therefore, cells undergoing apoptosis in the bursa are derived from rapidly dividing precursors.

Whereas the bursa is clearly critical for the establishment of a normal B cell compartment in the periphery, the progression of B cell development in the bursa remains unclear. However, we have defined a small population of cells, ~4% of the total, that express high levels of MHC class II and chL12 (a 40-kD common leukocyte alloantigen [18]), a phenotype indistinguishable from that of recent bursal emigrants in the periphery. We have suggested that this population may represent the most mature bursal cells. This population contains fewer cells in the S, G_2/M phases of the cell cycle compared with the remaining bursal cells and may therefore represent cells completing a cell cycle before emigration (13). Cells expressing high levels of the chL12 antigen contained <0.5% cells with <2 N DNA content (Fig. 3 *b*). Apoptotic cell death is therefore not random among bursal subpopulations. This conclusion provides further support that the chL12^{hi} population contains mature bursal cells that have survived selective events leading to bursal cell death and are committed to emigrate to the periphery.

Loss of Surface Ig Expression on Bursal Cells Undergoing Apoptotic Cell Death. Since apoptotic cell death was clearly not randomly distributed among bursal lymphocyte subpopulations, we analyzed the expression of a panel of cell surface markers on apoptotic (containing <2 N DNA) and nonapoptotic (containing ≥2 N DNA) bursal lymphocytes. This analysis revealed that entry into the apoptotic pathway did not result in changes in the levels of chB6 (Fig. 4, *a* and *d*), and MHC class II (Fig. 4, *b* and *e*). The same result was obtained using a panel of 40 other monoclonal antibodies against bursal cell surface antigens (data not shown).

In contrast, surface Ig expression was markedly reduced on bursal cells containing <2 N DNA (Fig. 4, *c* and *f*). This observation was confirmed using several anti-μ and anti-light chain mAbs that recognize nonoverlapping sites on bursal surface Ig. To formally conclude that the chB6$^+$, MHC class II$^+$ cells containing <2 N DNA were indeed B lineage cells, since some bursal macrophages express chB6 (22), we analyzed the purified DNA from sorted apoptotic cells for the presence of Ig gene rearrangements. All chicken B cells use unique functional Ig heavy and light chain V region genes (V_H1 and V_L1, respectively) and unique J sequences (J_H and J_L, respectively). Therefore all rearranged V(D)J genes can be amplified with unique PCR primer combinations 5′ to the V gene and 3′ to the J segment. Thus amplification of bursal DNA (>98% B lineage cells) revealed PCR amplified bands corresponding to rearranged V_H1 and V_L1 genes (Fig. 5). Amplification of DNA from sorted ex vivo apoptotic cells (>99% containing <2 N DNA) similarly revealed bands corresponding to both rearranged V_H1 and V_L1 genes.

The intensity of the PCR bands corresponding to rearranged V_H1 and V_L1 genes was lower than the equivalent bands from whole bursal cells. To determine whether this was a consequence of the fragmentation of DNA in apop-

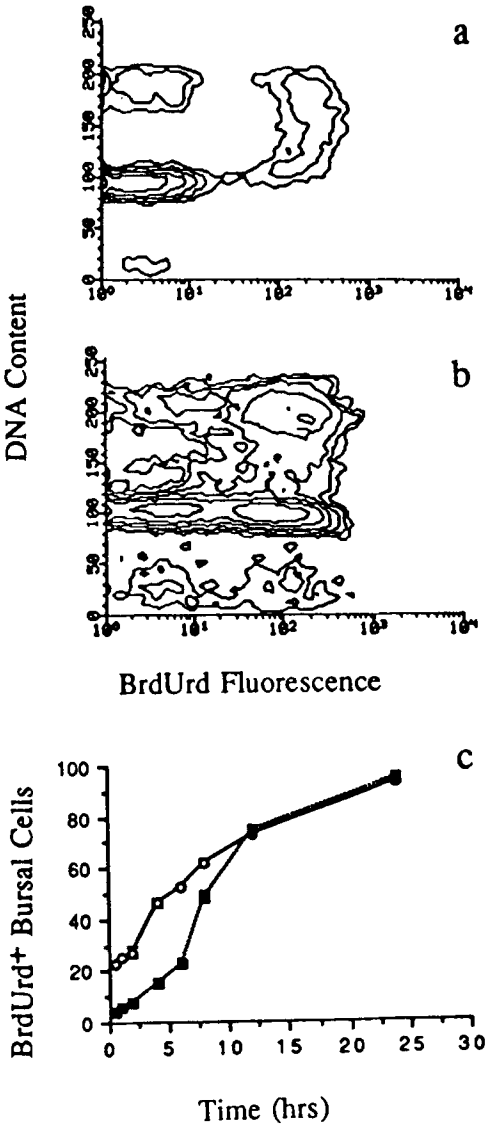

Figure 2. Apoptotic bursal cells arise from dividing precursors. 3-wk-old chickens were injected intraperitoneally with BrdUrd and the incorporation of the nucleotide analogue in bursal lymphocytes was detected by flow cytometry. Representative stains of bursal cell suspensions are shown for 1 h (*a*) and 8 h (*b*) after injection of BrdUrd. Kinetics of appearance of BrdUrd-labeled cells containing ≥2 N DNA (*open circles*) and <2 N DNA (*solid squares*) are shown in (*c*). Displayed are means ± SD of four chickens/data point.

totic samples, rearranged V(D)J genes were amplified from bursal cells induced to undergo apoptosis in vitro. Like most developing lymphocytes, bursal cells cultured in vitro rapidly undergo apoptotic cell death, with <10% cell viability after an overnight culture (23). After 8 h of culture, the apoptotic population, which contained <2 N DNA, represented >50% of the total cells (Fig. 6 *a*) and was flow cytometrically cell sorted on the basis of DNA content and chB6 expression to contain >99% B cells with <2 N DNA content. Amplification of V(D)J genes from this apoptotic population demonstrated bands corresponding to rearranged V_H1 and V_L1 genes. The intensities of the rearranged bands were similar to those observed with ex vivo apoptotic DNA, and considerably weaker than that observed with intact bursal DNA. Thus we conclude that despite the reduced efficiency with which Ig genes can be amplified from the fragmented DNA in apoptotic samples, those ex vivo apoptotic cells expressing little or no cell surface immunoglobulin are indeed B cells.

It was possible that entry into apoptosis induced a selective downregulation of cell surface Ig. Should this be the case, surface Ig would be lost from the surface of bursal cells undergoing apoptosis, irrespective of the mechanism by which the apoptosis is induced. Bursal cells cultured for 8 h in vitro contained >50% of apoptotic cells with <2 N DNA content. Cell counts revealed no significant reduction in cells excluding trypan blue during this period. There was no reduction of the expression of either surface Ig (Fig. 6, *c* and *e*) or of the chB6 antigen (Fig. 6, *b* and *d*) in this apoptotic population compared with cells from the same culture containing ≥2 N DNA. We conclude, therefore, that the loss of surface Ig among in vivo bursal cells with <2 N DNA must occur independent of the induction of apoptosis. Nonetheless, since all apoptotic cells in vivo have lost surface Ig expression we can further conclude that this loss preceded the induction of apoptosis. This suggests that the maintenance of surface Ig expression is required for the continued progression of B cell development in the bursa and that loss of surface Ig expression may trigger bursal cell apoptosis.

Since apoptotic bursal cells are derived from rapidly dividing precursors (Fig. 3), we examined the expression of bursal surface Ig during S phase of the cell cycle. Bursal cells in the first quarter of S phase (containing 2–2.5 N DNA) expressed relatively homogeneous high levels of surface Ig (Fig. 7 *c*). This suggests that entry into S phase may require surface Ig expression. However, bursal cell surface Ig levels became more heterogeneous as the cells progressed through S phase. Typically about 30% of the cells in the final quarter of S phase (containing 3.5–<4 N DNA) expressed lower mean surface Ig levels (Fig. 7 *c*). Although this population of cells did not resolve as a separate peak on the flow cytometric profiles, the shoulder of Ig^{lo} cells was completely reproducible in nine independent experiments. In contrast, levels of chB6 remained constant during S phase (Fig. 7 *b*). Bursal cells undergo a progressive enlargement during S phase (Fig. 7 *a*); therefore the reduction in membrane Ig levels in the surface Ig^{lo} population represents an even greater reduction in surface Ig density.

Since entry into S phase appears to require surface Ig expression and the levels of surface Ig density become heterogeneous as cells progress through S phase, it is possible that the surface Ig^{lo} cells observed late in S phase might represent precursors to the surface $Ig^{lo/-}$ apoptotic bursal cells. This is supported by the observation that apoptotic bursal cells are derived from dividing precursors (Fig. 3). To determine if bursal cells must complete a cell cycle before entering the apoptotic pathway, we arrested bursal lymphocyte division at metaphase with vincristine sulfate (16). Despite the accumulation of cells containing 4 N DNA 4 h after injecting vincristine, the proportion of lymphocytes in the apoptotic population was not reduced (Fig. 8 *a* and *b*). Taken together

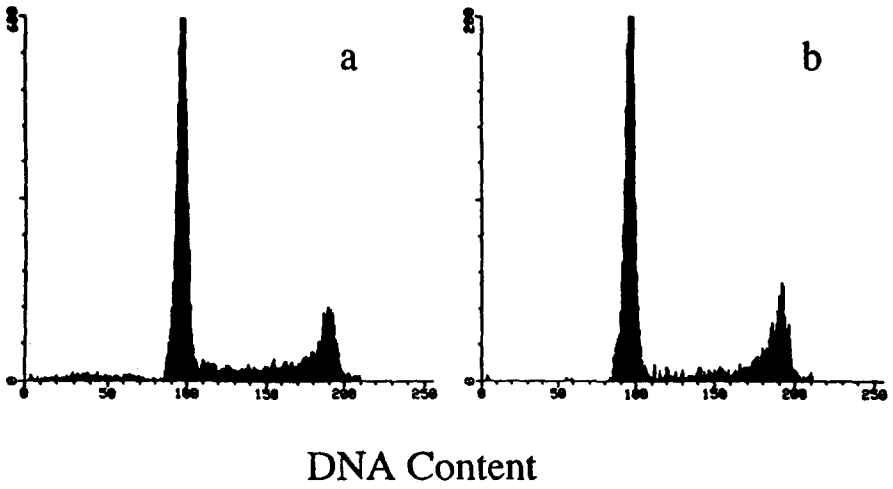

Figure 3. Apoptosis in the bursa is not random. Whole bursal cell suspensions (*a*) or cells gated for high levels of surface chL12 expression (*b*) were analyzed for DNA content. Proportions of cells with <2 N DNA for (*a*) 5.5 ± 0.3% and (*b*) 0.3 ± 0.1% were calculated from three chickens. 10,000 events are displayed gated on forward and side scatter.

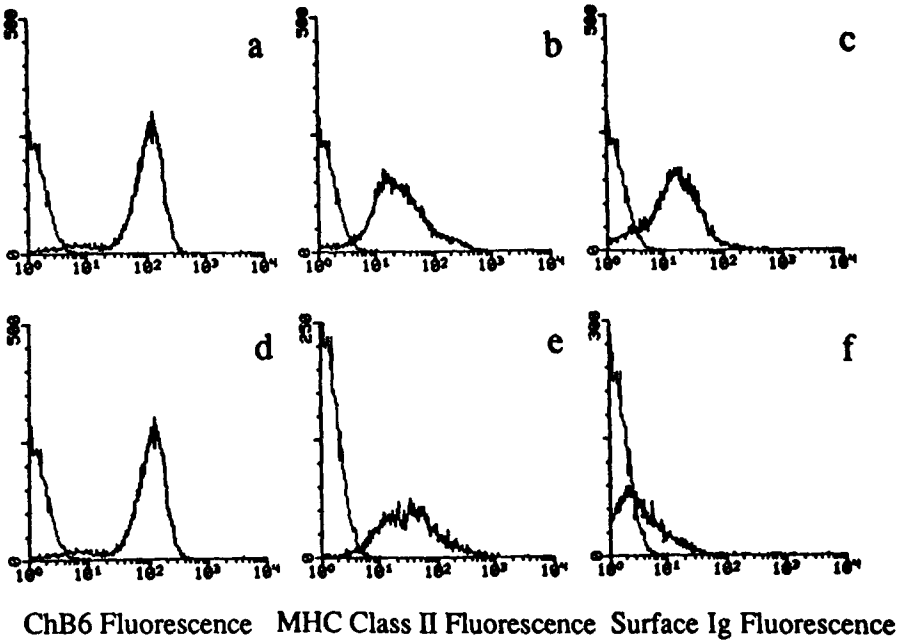

Figure 4. Phenotype of apoptotic ex vivo bursal lymphocytes. Cells containing ≥2 N DNA (*a–c*) and <2 N DNA (*d–f*) ex vivo bursal lymphocytes were analyzed for expression of (*heavy lines*) chB6 (*a* and *d*), MHC class II (*b* and *e*), and surface Ig (*c* and *f*), in comparison to a negative control antibody (*light lines*). 10,000 events are displayed gated on DNA content, forward and side scatter.

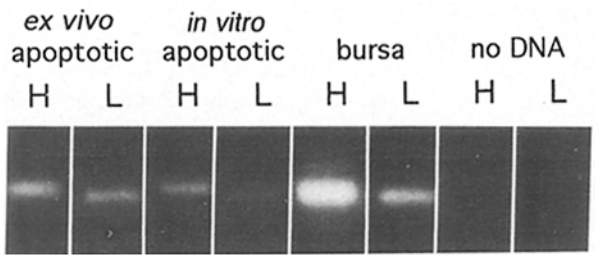

Figure 5. Ig V gene rearrangement in apoptotic bursal cells. Rearranged V_H1 (H) and V_L1 (L) genes were PCR amplified from DNA extracted from: ex vivo bursal cells purified by cell sorting to contain >99% chB6$^+$ cells with <2 N DNA content (ex vivo apoptotic), bursal cells cultured for 8 h in vitro before purification by cell sorting to contain >99% chB6$^+$ cells with <2 N DNA content (in vitro apoptotic), or unfractionated bursal cells (bursa).

with the results in Fig. 3, this demonstrates that apoptotic cell death is not restricted to a post mitotic population.

Discussion

We and others have previously shown by comparison of the rates of cell division and rates of emigration of B cells from the bursa that most newly generated bursal cells die in situ (8, 13, 15). We have confirmed that this death is apoptotic by the flow cytometric demonstration of cells containing <2 N DNA in bursal cell suspensions and by the presence of later stage condensed nuclei detected in phagocytes by electron microscopy. It is clear, therefore, that apoptotic cells in the bursa are eliminated in situ by phagocytosis, as is the case

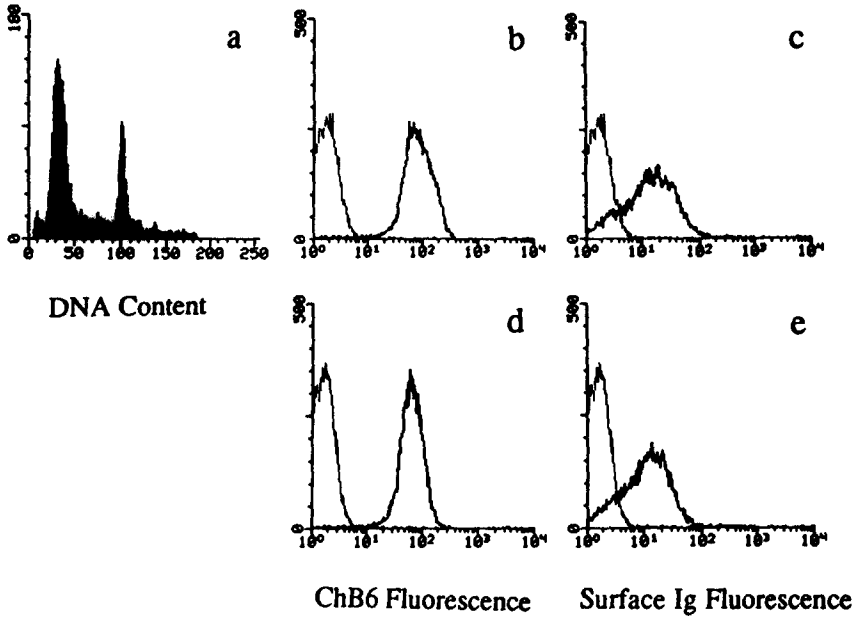

Figure 6. Surface Ig expression on apoptotic bursal cells after in vitro culture. Bursal lymphocyte suspensions were cultured for 8 h at 37°C. Cell cycle analysis showed about 55% of the cells containing <2 N DNA (*a*). Comparison of chB6 (*b* and *d*) or surface Ig (*c* and *e*) (*heavy lines*) expression between cells containing <2 N DNA (*d* and *e*) and cells containing ≥2 N DNA (*b* and *c*). Staining with negative control antibodies are shown by *light lines*. 10,000 events are displayed gated on DNA content, forward and side scatter.

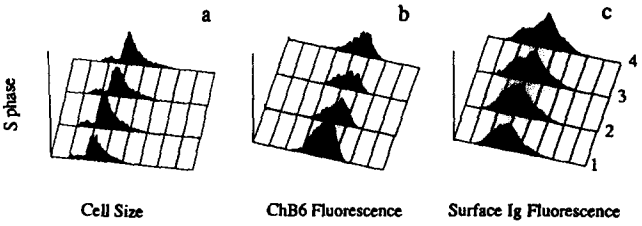

Figure 7. Surface Ig expression on bursal cells during S phase. Ex vivo bursal cell suspensions were stained for surface Ig expression and cellular DNA content. 250,000 events, gated on forward and side scatter, were acquired by flow cytometry. Cells undergoing S phase were divided during analysis into four successive quarters (>2–2.5 N DNA [1], 2.5–3 N DNA [2], 3–3.5 N DNA [3], and 3.5–<4 N DNA [4]) and analyzed for size (*a*), expression of the control marker chB6 (*b*), and surface Ig (*c*).

with bone marrow lymphocytes during murine B cell lineage development (24).

The bursa contains a substantial number of nonlymphoid cells, predominantly, but not exclusively, distributed in the medulla of bursal follicles. We have observed by electron microscopy phagocytic cells containing apoptotic nuclei in both the bursal follicular cortex and medulla (data not shown), confirming previous observations of tingible body macrophages in both sites presumed to contain apoptotic nuclei (8). The physiology of cortical and medullary bursal cells differ markedly in their rates of cell division (15, 16), although at this stage the functional basis of this distinction is unclear. Bursal lymphocyte cell death by apoptosis, however, can clearly occur in both anatomical compartments.

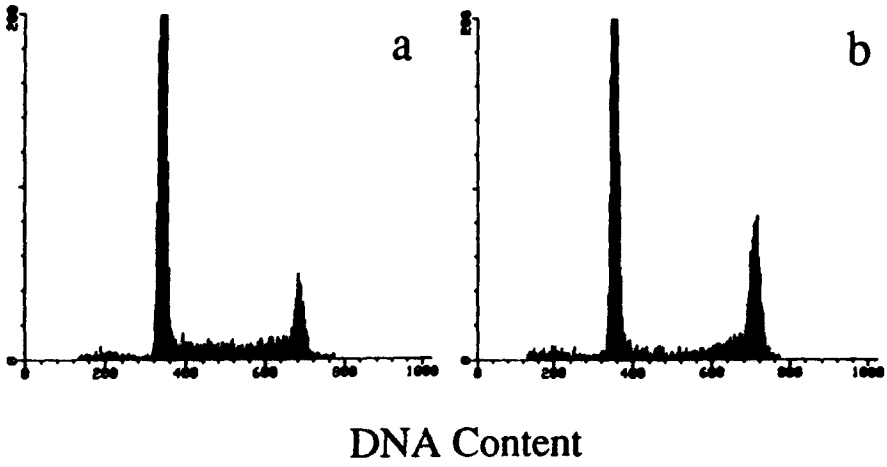

Figure 8. Metaphase arrest does not block induction of apoptosis. 5-wk-old chickens were injected intravenously with vincristine sulphate for 4 h and bursal cells were analyzed for DNA content, a representative profile of which is shown in (*b*) compared with age-matched control (*a*). 10,000 events are displayed gated on forward and side scatter and by the exclusion of cell doublets. Proportions of cells containing <2 N DNA, and in G1, S, and G2/M phases of the cell cycle were: 2.8 ± 0.4, 70.5 ± 0.3, 15.5 ± 0.1, 10.7 ± 0.2 for (*a*), and 3.3 ± 0.2, 61.8 ± 0.1, 11.6 ± 0.1, 22.3 ± 0.2 for (*b*), respectively, average ± SD from three chickens.

We show here that bursal cells cultured in vitro undergo apoptosis without losing surface Ig expression. Therefore the induction of apoptosis per se does not affect bursal cell surface Ig levels. However, surface Ig expression is markedly decreased in bursal cells undergoing apoptosis in vivo. Consequently, loss of surface Ig expression must occur independently of the induction of apoptosis. Since cells expressing low to negligible levels of surface Ig go on to die by apoptosis and all apoptotic cells isolated from the bursa express low to negligible levels of surface Ig, this suggests a causal relationship between lack of surface Ig and induction of apoptosis. Similarly, the maintenance of surface Ig expression is required for continued bursal cell growth and maturation.

In murine bone marrow, a similar requirement for expression of surface Ig is manifested; the inability to express a cell surface μ containing complex aborts B cell development at the pre-B cell stage (25). Additionally, B lineage cells in SCID mice, which do not functionally rearrange Ig genes and thus cannot express surface Ig complexes, also abort at the pre-B cell stage (26). In the murine bone marrow, deletion of B lineage cells occurs by apoptosis, as evidenced by the extensive numbers of condensed nuclei detected in the bone marrow by electron microscopy (27). Therefore, while it is likely that murine B cell precursors that have accumulated nonproductive rearrangements are eliminated by apoptosis, this has not been formally demonstrated.

Previous results have demonstrated that the bursa specifically potentiates those B cells which have undergone productive gene rearrangement during embryonic development (28). Consequently, bursal cells that have undergone nonproductive gene rearrangements will be eliminated. However, all rearrangement of chicken Ig genes occurs during the embryonic stage of development and all B cells in the hatched chicken are derived from those precursors which expressed surface Ig before hatch. Therefore, the lack of surface Ig expression on apoptotic bursal cells cannot be due to aberrant gene rearrangement events.

Somatic diversification of mammalian Ig genes occurs by point mutation during antigen driven B cell division in germinal centers (29) which are also a site of B cell death by apoptosis (30). While it is likely that those germinal center B cells which die by apoptosis are those which have accumulated point mutations that either disrupt the expression of surface Ig or reduce the affinity of the expressed Ig receptor for antigen presented by dendritic cells within the germinal center, formal proof has not been presented.

Ig diversity in the bursa is generated subsequent to productive gene rearrangement by gene conversion events in which sequences derived from upstream pseudo V_L or V_H genes replace the homologous sequences within the unique rearranged V_L1 or V_H1 genes (31, 32). This process appears to be independent of antigen (33). In a cell line (DT40) that continues to undergo gene conversion events in vitro, some gene conversion events can result in target V gene sequences becoming nonfunctional by the introduction of frame shifts or inappropriate stop codons (34). Consequently there is precedent for surface Ig^+ to Ig^- transitions occurring during somatic diversification by gene conversion. In addition, it is possible that gene conversion events may generate combinations of heavy chain and light chain V regions that are unable to combine into a functional Ig molecule. Nonproductive gene conversion events, therefore, could result in progressive loss of bursal surface Ig as the cell turns over the surface Ig expressed before such a gene conversion event.

At this point we have not determined what proportion of surface Ig^{lo}/apoptotic bursal cells contain such nonproductive gene conversion events. PCR analysis of DNA from ex vivo apoptotic cells revealed heavy and light chain V gene rearrangements (Fig. 5). In a separate experiment we have individually purified the bands of low molecular weight DNA from ex vivo bursal cells, generating a series of fractions with average sizes of 180, 360, 540 bp, etc. and detected by PCR rearranged Ig V genes. Critically, however, we demonstrated heavy and light chain rearranged V_H1 and V_L1 gene products of ~420 and ~350 bp, respectively, after PCR amplification from all fractions including the 180-bp apoptotic fragments (data not shown). The demonstration of PCR amplified products larger than the starting DNA fragment size demonstrates the occurrence of chimeric V gene products in the PCR. This suggests that the fragmentation of DNA from surface Ig^{lo}/apoptotic bursal cells will preclude unequivocal analysis of the sequence of the rearranged V genes. It should be emphasized however that the presence of chimeric rearranged V gene products does not invalidate this approach for the detection of gene rearrangement since the generation of chimeric products still requires V gene rearrangement detected by the VH5′/JH3′ or VL5′/VL3′ primer combinations.

The block of murine B cell development at the pre-B cell stage may be due to the absence of surface Ig-mediated signaling events that are crucial for continued B cell development (35). Ligation of bursal lymphocyte surface Ig complexes results in phosphoinositide hydrolysis and intracellular Ca^{2+} mobilization (17), and so bursal lymphocytes that have lost surface Ig would also lose the ability to receive surface Ig-mediated signals. Analogous to the development of murine B cell precursors, those bursal cells which express less than a threshold level of surface Ig required for continued B cell development would undergo programmed cell death. Therefore, loss of surface Ig would be a prelude to programmed cell death.

It is likely that the expression of surface Ig on bursal cells (or murine pre-B cells) involves recognition of the complex by an extracellular ligand. This ligand would need to be expressed in the bursa possibly on the surface of bursal stromal cells. We have previously argued that nonclonally distributed components of the surface Ig receptor are more likely to be involved in the recognition of a bursal ligand (36) than are the multiple different combining sites involved in a diversifying B cell repertoire. This leads to the prediction that introduction of a transgenic Ig heavy and light chain that could not undergo gene conversion, into bursal lymphocytes whose endogenous Ig genes are undergoing gene conversion should rescue from apoptosis those cells whose endogenous surface Ig is lost after nonproductive gene conversion events.

Bursal cells entering S phase have relatively homogeneous and high levels of surface Ig, despite the presence of cells with a 2 N DNA content (G_0/G_1) expressing low levels of surface Ig. This suggests the possibility that bursal cell DNA replication is regulated, at least in part, by the presence of threshold levels of surface Ig and therefore surface Ig-mediated signals may be required for entry into S phase of the cell cycle.

During the second half of S phase about 30% of cells begin to express lower levels of surface Ig, suggesting that the event(s) causing this loss have occurred early in S phase. In this regard, it is widely assumed that gene conversion events in bursal Ig diversification occur among the rapidly dividing B cell population (e.g., 37). The failure of metaphase arrest to block the appearance of cells with <2 N DNA suggests that apoptosis may occur in S/G2/M, in other words without a requirement for completion of a cell cycle. This is precedented in that murine thymic T cells activated in vitro also undergo apoptosis during S phase of the cell cycle (38).

It is likely that nonproductive gene conversion events are not the only cause of cell death in the bursa. We have demonstrated that two populations of bursal cells emigrate to the periphery (13, 15, and Paramithiotis, E., and M. J. H. Ratcliffe, manuscript in preparation). The major population of bursal emigrants is directly derived from the rapidly dividing cells in the bursal cortex and may represent a naive repertoire of B cell specificities analogous to newly generated bone marrow–derived murine B cells. The fate of those bursal cells in this naive repertoire which recognize self-antigens is unclear. In murine bone marrow at least some developing B cells are eliminated based on surface Ig receptor occupancy and degree of surface Ig cross-linking (39, 40). Consequently extensive cross linking of the surface Ig on this population of bursal cells may induce deletion of self reactive B cells possibly by apoptosis.

The second population of bursal emigrants to the periphery contains cells with an extended lifespan that do not undergo further cell division in the periphery (13). We have suggested that these cells may be derived from the bursal medulla, an environment that contains gut-derived antigens, and as a result this repertoire may be enriched for those specificities that have encountered extrinsic antigen in the bursa. It is therefore possible that some bursal cell death is a consequence of bursal cells in the bursal medulla failing to encounter antigen and consequently failing to be selected into the long-lived bursal emigrant population.

In conclusion, only a minority of newly generated bursal cells emigrate to the periphery; the remainder die by apoptosis in situ. We show here that bursal lymphocyte death is related to the levels of surface Ig expression, but that the induction of apoptosis does not itself induce loss of surface Ig expression. We conclude, therefore, that the progression of chicken B lymphocyte development requires continued surface Ig expression and those cells which lose surface Ig are eliminated by apoptosis.

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