

Induction of apoptosis during normal and neoplastic B-cell development in the bursa of Fabricius

(programmed cell death/tumor progression/*myc* oncogenes/radiation)

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ABSTRACT The lymphoid cells of embryonic bursal follicles are engaged in rapid growth and preimmune diversification of immunoglobulin genes. Disruption of follicular architecture by mechanical dispersion of these cells in short-term tissue culture was accompanied by continued cell division and extensive cell death by apoptosis. Apoptosis was suppressed in parallel cultures of intact follicles. γ Radiation also triggered extensive apoptosis in embryonic bursal follicles within a few hours. Preneoplastic bursal stem cell populations induced by a *v-myc* oncogene were hypersensitive to induction of apoptosis by follicular dispersion and radiation. In contrast, tumor progression in *v-myc*- and *v-rel*-initiated bursal neoplasms was accompanied by development of resistance to induction of apoptosis. A programmed cell death pathway can be activated during normal B-cell development in the bursa, and alterations in the expression of this pathway accompany neoplastic change in this system.

Programmed cell death or apoptosis is now widely recognized as a common mechanism for selective elimination of cells during development of many animal cell lineages. This process requires RNA and protein synthesis and leads to a dramatic condensation of the cell nucleus associated with extensive intranucleosomal cleavage of nuclear DNA (1). In the immune system much work on programmed cell death in the thymus and various T-cell-derived lines has been reported (reviewed in refs. 2 and 3), but less extensive data are available concerning the possible role of apoptosis in B-cell development. As with T cells, some factor-dependent neoplastic B-cell lines will undergo apoptosis upon either withdrawal of the required growth factor (4, 5) or cross-linking of surface immunoglobulins (6, 7) and other surface molecules (8) with antibodies. Human B-cell leukemias (9) and murine splenocytes (10) die rapidly by this process if placed in short-term tissue culture. An interesting set of recent observations indicates that overexpression of the oncogene *Bcl-2* specifically inhibits apoptosis in murine splenocytes and factor-dependent B-cell lines (4, 5).

We have found recently that developing B-cell populations in the avian bursa of Fabricius are highly susceptible to programmed cell death. Extensive apoptosis was rapidly induced in normal embryonic bursal cell populations either by physically disrupting cell contacts within bursal follicles or by exposure to γ radiation. Preneoplastic bursal stem cell populations induced by a *myc* oncogene were hypersensitive to induction of apoptosis, whereas more advanced neoplastic bursal cell populations derived from these preneoplastic cells were very resistant to induction of apoptosis by these stimuli. These observations suggest that the regulation of the pathway leading to programmed cell death may be an important feature of B-cell development in the bursa and that alterations

in that regulation accompany neoplastic change in this system.

MATERIALS AND METHODS

Bursal Cell Populations. All bursal cells came from inbred White Leghorn line SC chickens (Hyline Farms, Dallas Center, IA). Normal large cycling embryonic bursal lymphoblasts (11) were obtained from bursal follicles between 18 days of embryogenesis and hatching.

Transformed follicles induced by the *v-myc* oncogene were obtained by infection of embryonic bursal follicle populations with the myelocytomatosis virus HB1 and transplantation into cyclophosphamide-treated recipient chicks as described (12). Cyclophosphamide selectively ablates the lymphoid cell populations of the recipient bursa (13). As we have reported previously, reconstitution with HB1-infected embryonic bursal cells generates up to 50% transformed follicles indistinguishable from the preneoplastic lesions seen in smaller numbers in standard avian leukosis virus-induced bursal lymphomagenesis (14-16). These *v-myc*-induced transformed follicles contained monomorphic populations of pyrimophilic lymphoblasts (TF cells) with the cardinal properties of bursal stem cells, including ongoing diversification of their immunoglobulin genes and efficient reconstitution of cyclophosphamide-ablated follicles in secondary bursal transplantation procedures (11). Virtually complete populations of transformed follicles were obtained by this method from secondary transplants (11, 17). Essentially homogeneous populations of TF cells were then prepared from these follicles for comparison with the normal cycling embryonic bursal populations.

As previously reported (11, 17), about 20% of the transplants with TF cells showed areas of invasive lymphoma formation that could be compared with the preneoplastic transformed cell population. In addition, cultured cell lines derived from *myc*-induced bursal lymphomas BK3A (18) and DT-40 (15) were analyzed. Finally, neoplastic bursal cell lines were established rapidly in culture by infection of normal bursal cells (line REVNB-1) and TF cells (line REVTF-1) with a *v-rel* transducing reticuloendotheliosis virus (REV) as described (19).

Follicle and Cell Preparation for Short-Term Culture. Bursas were dissected from the peritoneal surface of the cloaca and opened to expose bursal folds in a Petri dish with 5 ml of RPMI 1640 medium supplemented with 3 μ M 2-mercaptoethanol, 2 mM glutamine, 1 mM sodium pyruvate, 0.35% tryptose phosphate, 8% fetal calf serum, and 10% chicken serum. Bursal follicles were released by scraping the bursal folds with a scalpel blade and the residual bursal tissue was discarded. The follicles were gravity sedimented twice in a conical test tube for 3 min to remove most detached cells. Follicles were cultured either intact for up to 24 hr in the same

medium or dispersed by passage of through a Dounce homogenizer with an "A" pestle followed by filtration through 40- μm Nitex mesh. Starting cell concentrations in dispersed cultures were adjusted to 10^6 per ml, and live cells were counted by hand in a hemocytometer in 0.4% trypan blue. For morphological analysis, aliquots (10^5 cells) of dispersed cultures were deposited on slides in a Shandon cytofuge and stained with Wright's stain.

Radiation and Histology. Radiation to whole birds was carried out in a calibrated ^{137}Cs irradiator. Bursas for histological examination were fixed in Carnoy's solution and stained with methyl green pylonin.

Cell Cycle Analysis. Bursal cell populations were suspended in a 0.1% Triton X-100 solution containing 4 mM sodium citrate, 30 Kunitz units of RNase per ml, and 50 μg of propidium iodide per ml (pH 7.8) and incubated for 10 min at 37°C. NaCl was then added to 14 μM , and the preparation was held at 4°C for analysis of cellular DNA content in a fluorescence-activated cell sorter. Computer-assisted estimation of the proportion of bursal cell populations in the G_1 , S, and G_2/M phases of the cell cycle employed the multicycle software developed by Peter Rabinovitch (Phoenix Flow Systems, San Diego).

Analysis of DNA Degradation. Bursal cells were suspended in RSB buffer (10 mM Tris-HCl/10 mM NaCl/5 mM MgCl_2 , pH 7.4) and mixed with an equal volume of lysis buffer (1% sodium dodecyl sulfate/20 μM Tris-HCl/100 mM KCl/10 mM EDTA/16 mM dithiothreitol, pH 7.4). Proteinase K was added to a final concentration of 0.5 mg/ml for at least 3 hr at 55°C. After addition of 0.5 vol of 7.5 M ammonium acetate, nucleic acids were precipitated with an equal volume of isopropyl alcohol, redissolved in 10 mM Tris-HCl/1 mM EDTA, digested for 30 min with 150 μg of ribonuclease A per ml, and then digested again with proteinase K overnight. Aliquots of this digest were analyzed by electrophoresis in mixed 0.7% SeaKem/1.1% NuSieve (FMC) agarose gels.

RESULTS

Induction of Apoptosis by Disruption of Cell Contacts in Bursal Follicles. Suspensions of bursal lymphocytes died in culture. Fig. 1A shows that suspensions of cells from normal embryonic bursal follicles, dispersed in short-term culture as described in *Materials and Methods*, begin to decrease in number after about 2 hr. Cycloheximide appeared to inhibit cell death in these cultures (Fig. 1B). Fig. 2 shows Wright-stained cytofuge preparations of these cells 4 hr after culture, with many dying cells with intact cytoplasm and markedly

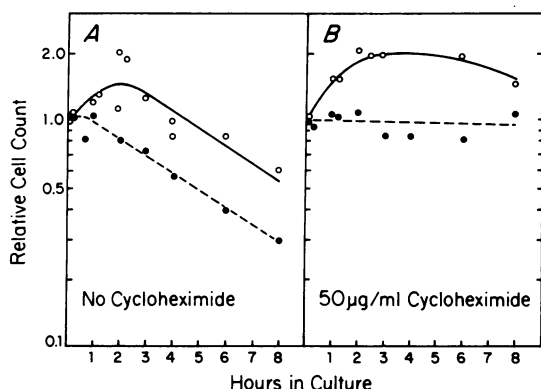


FIG. 1. Viable cell counts of normal embryonic bursal lymphoblasts (\circ) and TF cells (\bullet) were obtained at various times after dispersion in short-term culture either without (A) or with (B) cycloheximide in the culture medium. The cell counts at zero time were set at 1.0 and the mean values of duplicate samples from two different experiments were normalized to the counts at zero time.

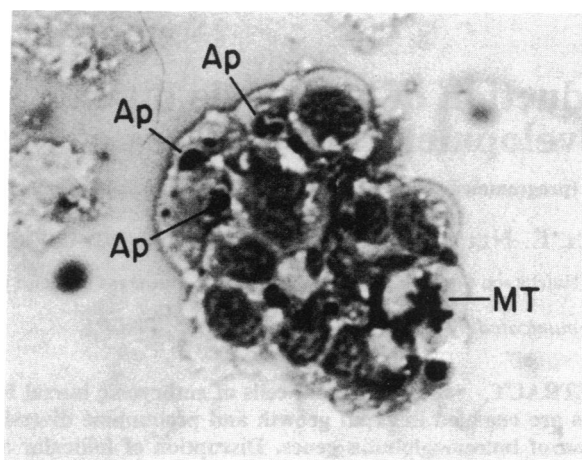


FIG. 2. Wright's stain of normal embryonic lymphoblasts 4 hr after dispersion in culture showing cells undergoing apoptosis (Ap) and mitosis (MT). ($\times 750$.)

condensed nuclei characteristic of apoptosis. DNA extracted from these cells 2 and 4 hr after dispersion in culture shows a pattern of degradation characteristic of extensive intranucleosomal cleavage (Fig. 3, lanes 2 and 4). In striking contrast, DNA from parallel bursal follicle cultures that were not subjected to the dispersion procedure showed far less evidence of DNA degradation (Fig. 3, lanes 3 and 5). These observations suggest that simple physical disruption of cell contacts in bursal follicles markedly stimulates programmed cell death (apoptosis) in these cycling (see Table 1) normal bursal lymphoblasts.

Inspection of the growth curve for dispersed normal embryonic bursal lymphoblasts (Fig. 1A) indicates that population growth continues for about 2 hr before cell death begins to dominate in the culture. At later times in the culture, as shown in Fig. 2, some cells continue to undergo mitosis even while others are dying by apoptosis. Within bursal follicles this population is rapidly proliferating. Analysis of partitioning in cell cycle compartments shown in Table 1 indicates a modest further shift of normal embryonic lymphoblasts into the S and G_2/M phases of the cell cycle after dispersion in culture. Therefore physical dispersion of the lymphoid and stromal cells of the follicle and/or induction of programmed

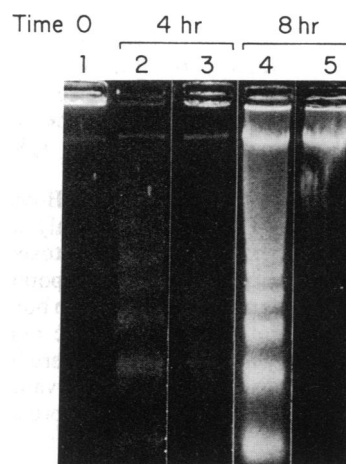


FIG. 3. Agarose gel electrophoresis of total cell DNA stained with ethidium bromide from normal embryonic lymphoblasts before culture (lane 1), after dispersion and culture for 2 and 4 hr (lanes 2 and 4, respectively), or after culture as intact follicles for 2 and 4 hr (lanes 3 and 5, respectively). The fastest migrating bands in lanes 2 and 4 are about 200 base pairs (markers not shown).

Table 1. Cell cycle analysis of bursal lymphoblast populations

Bursal cell type	Radiation dose, R	Time,* hr	n [†]	Fraction of cells [‡]		
				G ₁	S	G ₂ /M
TF cells [§]	0	0	4	0.57	0.35	0.08
Embryonic lymphoblasts	0	0	8	0.60	0.31	0.09
	0	2	8	0.50	0.37	0.13
	160	2	2	0.41	0.43	0.16
	160	4	2	0.40	0.29	0.31

*Time either in short-term culture or, for the irradiated bursas, after treatment.

[†]n, Number of bursas analyzed.

[‡]Fraction of cells in each compartment of the cell cycle was determined by measurement of DNA content.

[§]*myc*-Induced preneoplastic bursal stem cells from TFs prepared as described in the text.

cell death in the population do not seem immediately to inhibit the cell division cycle.

The preneoplastic bursal stem cells of TFs (TF cells) are also large cycling lymphoblasts with cell cycle characteristics similar to those of normal embryonic populations (Table 1). Dispersion of these cells in culture also triggers the onset of cell death, but with a shorter delay (by about 2 hr) than seen in normal cell cultures (Fig. 1). TF cell death was also inhibited by cycloheximide (Fig. 1), and the morphologic features and DNA degradation pattern characteristic of apoptosis were also seen in cultured TF cells (not shown). The basis for this shorter delay period in TF cells remains to be determined.

Radiation-Induced Apoptosis. γ Irradiation induced cell death by apoptosis in the bursa. Fig. 4B shows the appearance of extensive apoptosis in a normal embryonic bursal follicles 4 hr after receiving 520 R of γ radiation. Table 1 shows that at lower doses of radiation—e.g., 160 R—normal embryonic bursal lymphoblast populations tended to accumulate in the G₂ phase of the cell cycle as described for most eukaryotic cells in response to DNA damage (reviewed in ref. 20). However, at radiation doses that triggered enzymatic DNA cleavage the cell cycle analysis could not be carried out with precision by this technique due to nuclear fragmentation. Thus, it is not yet

Table 2. Radiation-induced apoptosis in the bursa

Parameter	Dose, R									
	40-120		160-200		280		400		520	
	NB	TF	NB	TF	NB	TF	NB	TF	NB	TF
n*	8	9	6	5	3	2	3	3	2	2
Med [†]	1+	2+	1+	3+	1+	4+	2+	4+	3+	3+
Range	0-1+	1+-3+	0-1+	2+-3+	1+-3+					

Birds were irradiated in a ¹³⁷Cs irradiator and after 4 hr bursas were obtained for histological evaluation. NB, normal bursal follicles at hatching; TF, TF from secondary transplantation procedures obtained 4 weeks after hatching.

*n, Number of bursas analyzed.

[†]Median score. A score of 1+ indicates an occasional pyknotic cell observed by examination under oil ($\times 1000$). Scores of 2+–4+ indicate increasing extents of apoptosis observed at low power ($\times 150$). Slides were read blind—i.e., without knowledge of radiation dose.

clear from these studies whether there was a cell cycle-regulated component to the induction of apoptosis.

Radiation also induced apoptosis in TF cells. When a dose–response relationship was examined, as shown in Table 2, we found that extensive apoptosis could be induced with as little as 160–200 R in *myc*-induced transformed follicles, whereas normal embryonic populations required about 500 R to produce equally extensive morphologic changes. This differential sensitivity to radiation-induced apoptosis was also detected by analysis of DNA degradation 4 hr after irradiation (Fig. 5). Oligonucleosomal DNA ladders were detected with as little as 200 R for TF cell populations and at 480 R for normal embryonic bursal lymphoblasts. Thus TF cells were relatively hypersensitive to radiation (i.e., triggered apoptosis with fewer DNA strand breaks).

Over the first few weeks after hatching normal bursal cell populations change dramatically. Small “resting” lymphocytes predominate, although large activated lymphoblasts are still present (9). The response to radiation was more complex in the normal posthatching bursa. We found that 4 weeks after hatching 480–520 R of γ irradiation would induce the morphologic changes of apoptosis, but principally among the large activated bursa lymphoblasts. The large majority of small

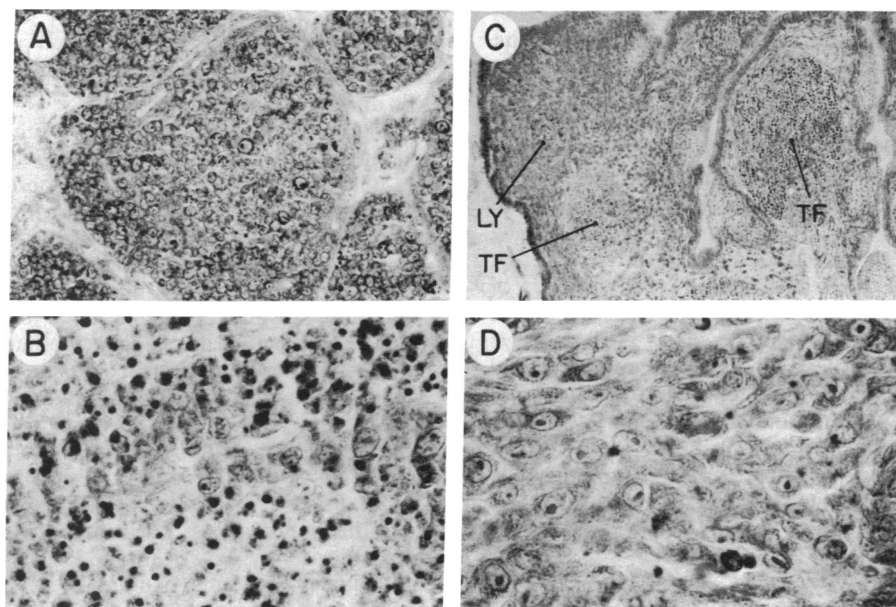


FIG. 4. Methyl green pyronin-stained histologic sections of normal 18-day embryonic bursal follicles (A, $\times 160$), normal embryonic bursal follicles and embryonic follicles 4 hr after 520 R (1 R = 0.258 mC/kg) of γ radiation (B, $\times 320$). (C) Low-power view ($\times 80$) of a bursa containing TFs showing extensive apoptosis and a lymphoma (LY) 4 hr after 520 R of γ radiation. (D) Higher magnification of the lymphoma ($\times 320$) showing the absence of apoptosis.

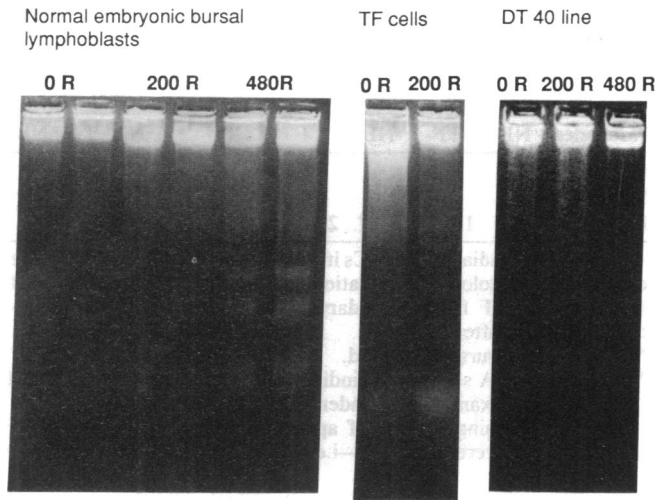


FIG. 5. Agarose gel electrophoresis of pairs of duplicate DNA samples from normal embryonic bursas 4 hr after 0, 200, and 480 R of γ radiation. DNA from TFs was analyzed 4 hr after 0 and 200 R. DNA from the DT-40 cell line was analyzed 4 hr after 0, 200, and 400 R.

resting cells seems resistant to this dose of irradiation (not shown).

Resistance to Induced Apoptosis in Neoplastic Bursal Cells. Fig. 4 shows a striking contrast between the response of TFs and a derivative bursal lymphoma to radiation. The preneoplastic TFs show almost total cell death with the features of apoptosis, whereas the neoplasm shows no morphologic effects. This apparent resistance to radiation-induced cell death by apoptosis is also illustrated in Fig. 5 by a bursal lymphoma-derived cell line still active in immunoglobulin gene conversion, DT-40 (19, 27). No increase in DNA degradation was detected following radiation in these cells. Very low levels of oligonucleosomal DNA degradation ladders (difficult to reproduce in photographs), however, were detected by inspection of agarose gels in all DNA preparations from these cells independent of exposure to radiation. We believe this represents a small amount of degradation occurring during the extraction procedure (which included Mg^{2+} -containing buffers). If so, the relevant nuclease(s) could well be present in such cells, implying that the block in the pathway leading to apoptosis might occur at a regulatory step preceding the activity of these enzymes.

We tested the sensitivity of a number of bursal-derived cell lines, in addition to DT-40, to various agents reported to induce apoptosis. These cells included other bursal lymphoma-derived lines and lines produced by direct infection of normal bursal cells and TF cells with a *v-rel* transducing retrovirus (19). The *v-rel*-transformed lines differed in that those derived from TF cells contained very high levels of HB1 viral *v-myc* mRNA, whereas those derived from normal bursal populations contained lower levels of normal *c-myc* mRNA (not shown). Regardless, all of these neoplastic bursal lines were resistant to induction of apoptosis by radiation as well as by 10 μ M dexamethasone and calcium ionophore A23187 at 125 ng/ml (not shown). Thus direct *v-rel* transformation of bursal lymphocytes and progression of preneoplastic to neoplastic stages during *myc*-induced multistage lymphomagenesis are accompanied by the suppression of programmed cell death.

DISCUSSION

Normal developing bursal lymphoblasts, as a population, undergo destruction by apoptosis within a few hours of either disruption of their normal cell contacts or exposure to

relatively low doses of ionizing radiation. This rapid suicidal response suggests that the pathway for programmed cell death and its regulation may be important in the control of B-cell development in the bursa. As in other developing systems where programmed cell death is active, however, the precise role of this process is not immediately obvious.

Considering the apparent principal functions of the bursa, at least two speculations about the role of programmed cell death suggest themselves. Both ideas assume that induced suicide serves as a fail-safe mechanism to eliminate errors detrimental to the organism. (i) Embryonic bursal lymphoblasts are very active in preimmune diversification of their immunoglobulin genes by a gene conversion mechanism (21, 22), and the resultant immunoglobulins are expressed on the cell surface. It is possible that the cell death response is an efficient mechanism for removal of cells expressing relatively defective immunoglobulins. The generation or lack of specific signals resulting from the presence of such defective molecules on the cell surface could trigger apoptosis, and our mechanical disruption of surface contacts could simply mimic this mechanism. In fact, it has been suggested that apoptosis in germinal centers of lymph nodes may be the mechanism for antigen-driven selection of immunoglobulins (23). (ii) The bursal environment is required for the rapid expansion of the developing B-cell mass in avians (24). Cell contacts or short-range associations within the architecture of the bursal follicle may serve to restrain and modulate the tremendous proliferative capacity of bursal lymphocytes. Induced apoptosis may be a vital mechanism to avoid potentially disastrous consequences of unrestrained growth of clones in which such contacts become defective, a circumstance, again, that could be mimicked by our disruption experiment. This growth control concept could, of course, apply to many of the developing systems in which the programmed cell death pathway is activated. Determination of which, if either, hypothesis is correct in the bursa requires further investigation.

Why and how DNA damaging agents such as radiation trigger apoptosis are also unknown. Whatever the mechanism, the relative sensitivity of the bursa to induction of programmed cell death by DNA-damaging agents is useful experimentally. Alkalating agents such as cyclophosphamide can permanently destroy the bursal lymphoid population at doses that do not harm the bursal stroma or irreversibly damage other proliferating organs and tissues (13). The bursal transplantation technique that we have employed exploits this differential sensitivity.

Progression from preneoplastic TF cells to full neoplastic phenotype requires acquisition of the ability to grow outside the follicular architecture. Thus suppression of an efficient cell death pathway triggered by separation of TF cells from the normal follicular environment should be a requirement for tumor progression to occur in this multistage system. This prediction was supported by the observations described in this study. It would be interesting to learn if this suppression is due to an active mechanism, perhaps common in B-cell neoplastic change such as could be mediated by *Bcl-2* or another oncogene, or simply from loss of function mutations in the cell death pathway. Using murine and human probes (generously supplied by Stanley Korsmeyer, Washington University) we have not detected expression of *Bcl-2* RNA in chicken bursal cell populations by low-stringency, blot-hybridization analysis, but these studies are not yet definitive. Apparently expression of *v-rel* in bursal lymphocytes also rapidly eliminates expression of the cell death pathway as a facet of acute transformation of these cells. Activation of Epstein-Barr virus latent genes has recently been reported to protect Burkitt lymphoma-derived cell clones from apoptosis (25), and oncogenic mutations in *ras* have also been reported to suppress apoptosis in neoplastic rodent fibroblasts (26).

The significance of the relative hypersensitivity of preneoplastic TF cells to apoptosis induced by follicular disruption or radiation is not so immediately apparent. These cells represent developmentally arrested bursal stem cells, whereas the normal lymphoblasts to which we compared them are largely the immediate progeny of bursal stem cells. Thus it is possible that the programmed cell death pathway is differentially regulated at these stages in bursal development. The obvious alternative explanation is that the pathway is influenced more directly by the massive overexpression of *v-myc* in TF cells (17). We have not observed evidence, however, that apoptosis in normal bursal lymphoblasts is directly regulated by *myc* expression. For example, we were unable to detect a significant change in the synthesis of MYC protein following either radiation- or disruption-induced apoptosis by radioimmune precipitation assays carried out in collaboration with Robert Eisenman's laboratory (data not shown). Whatever the mechanism, a final speculation concerning the role of events in the cell death pathway in neoplastic change concerns the possibility of nonlethal DNA damage acquired by this mechanism. Although no direct evidence is available showing the occurrence of such damage, repair and/or recombinational responses resulting from such events could contribute to genetic events mediating neoplastic progression in preneoplastic cell populations.

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