A retroviral *myc* gene induces preneoplastic transformation of lymphocytes in a bursal transplantation assay

(avian myelocytomatosis virus/*Blym*-1/bursal transplantation/transfection)

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ABSTRACT Treatment of chicken embryos with cyclophosphamide results in ablation of bursal lymphocytes. Bursal follicles can be reconstructed by infusion of embryonic bursal cells. Histologic examination of reconstituting bursal follicles showed that the first lymphocytes to appear were large pyrinophilic lymphoblasts that lined up adjacent to the bursal basement membrane and appeared to serve as progenitors for the differentiation of bursal medullary lymphocytes. When these cells were infected with the avian myelocytomatosis virus HB1 bearing a *v-myc* oncogene they appeared to home to the region of the bursal basement membrane but failed to differentiate. Instead, they formed structures indistinguishable from the preneoplastic transformed follicles that develop during bursal lymphomagenesis induced by lymphoid leukemia viruses. The DNA from these transformed follicles contained the HB1 *v-myc* gene but lacked the ability to transform NIH/3T3 mouse cells. Therefore these preneoplastic lesions were induced directly by HB1 *myc* and did not require the expression of *Blym*-1 or similar oncogenes. Exploitation of this transplantation technique with the chicken bursa will provide a useful method for assessing the stage-specific activity of oncogenes *in vivo*.

The activation of cellular oncogenes in a variety of human and animal tumors has fostered the hypothesis that these genes are involved in the initiation and/or progression of naturally occurring neoplasms (see ref. 1 for recent reviews). Although the tumor-specific activation of these genes has provided a strong circumstantial argument for their involvement in the pathogenesis of neoplasms, direct experimental demonstration of the role of oncogenes in tumorigenesis has been lacking. Furthermore, the specific role of individual oncogenes in tumor development is not known. We present here an *in vivo* experimental approach to these questions, utilizing the technique of embryonic bursal transplantation. With this technique it is possible to reconstruct bursal follicles that have been ablated by the alkylating agent cyclophosphamide by transplantation of a small number of embryonic bursal lymphocytes. This approach has allowed us to examine, histologically, the events that are involved in reconstitution of the bursal follicles and to determine the effects of the introduction of a viral oncogene, *v-myc*, on this process. These studies have provided the opportunity to observe the phenotype of bursal lymphocytes containing this oncogene *in vivo*. They also suggest which normal cells in the bursa serve as targets for transformation by activated *myc* oncogenes.

These studies in the chicken bursa are relevant to the issue of the role of cellular oncogenes in tumorigenesis. Lymphoid leukemia virus (LLV) induces bursal lymphomas that contain two distinct tumor-specific oncogenes: *c-myc*, which is tran-

scriptionally activated as a result of nearby integration of the LLV provirus (2, 3), and *Blym*-1, which transforms NIH/3T3 mouse cells in transfection assays (4-6). Human homologs of both *c-myc* (7-9) and *Blym*-1 (10) are also reproducibly activated in a human B-cell neoplasm (Burkitt lymphoma). In LLV-infected chicken bursas these lymphomas are preceded by preneoplastic lesions, called transformed follicles, composed of pyrinophilic lymphoblasts (11, 12). Nothing is known about the activation of oncogenes at this early stage of lymphomagenesis.

MATERIALS AND METHODS

Chickens. Embryos from specific pathogen-free (SPF) White Leghorn chickens were obtained from H & N Farms, Redmond, WA. Most were from an inbred line developed originally from Kimber Farm line 28 (K28 stock) contributed by Harriet Robinson.

Bursal Transplantation. Chicken embryos were injected with 1.25 mg of cyclophosphamide in 0.1 ml of sterile isotonic saline in a chorioallantoic vein on days 15, 16, and 17 of development. About 1×10^6 embryonic bursal cells from 18-day embryo donors were injected in 0.1 ml of Dulbecco's modified Eagle's essential medium (DME medium) on day 18. For preparation of donor bursal cells, bursas were removed from 18-day embryos under sterile conditions, placed in 5 ml of DME medium in a tissue culture plate, and opened with a scalpel. The bursal folds were scraped with the scalpel blade to release follicles. The bulk of the stroma was discarded. A single-cell suspension of bursal lymphocytes was obtained by one or two strokes of a Dounce homogenizer. The bursal cells were then filtered through nylon mesh and pelleted by centrifugation prior to resuspension and infection with the appropriate virus. After hatching, reconstituted chickens were housed in barrier isolators for 4 weeks. Birds were sacrificed electively or when they appeared to be sick at various times after hatching. Bursas were prepared in Carnoy's fixative and embedded in paraffin. Sections were stained with methyl green pyronin (MGP).

Viruses and Infection. Myelocytomatosis virus HB1-(tdB77) was propagated and titered on quail embryo fibroblasts. Virus stocks of HB1 were concentrated to titers near 10^8 focus-forming units per ml in a Pellicon cassette system and stored at -80°C until use. Infection of bursal cells was carried out at 37°C for 30 min in the presence of Polybrene at $2 \mu\text{g/ml}$ at a multiplicity of infection of 1-10. Infected bursal cells were washed once in Tris-buffered saline before suspension in DME for injection into recipient embryos.

Extraction of DNA and Blot Hybridization Analysis. Nuclear DNA was analyzed from lymphocytes obtained from reconstituted bursas (approximately 1×10^8 cells). The tech-

nique for DNA extraction, restriction endonuclease digestion, and agarose gel electrophoresis transfer to nitrocellulose sheets followed the technique of Southern (13). Probes for blot hybridization analysis were exon and intron sequences of the *c-myc* locus separately subcloned in pBR322 (see Fig. 2). These plasmids were labeled by nick-translation and annealed with the DNA on the nitrocellulose membrane at 42°C in hybridization buffers containing 50% formamide (vol/vol) and 10% dextran sulfate.

Assay of Transforming Genes in Bursal DNA. High molecular weight (greater than 20×10^6) DNAs were assayed for focus formation by transfection of NIH/3T3 mouse cells as

previously described (14) except that dexamethasone was not included in the culture medium.

RESULTS

Experimental Strategy. We have investigated the effect of an activated *myc* gene on bursal lymphocytes *in vivo* by exploiting an embryonic bursal transplantation technique based on the work of Eskola and Toivanen (15). The strategy we used was to introduce the activated *myc* gene associated with the recovered avian myelocytomatosis virus HB1 into bursal stem cells by simple infection and then to transplant

FIG. 1. Sections of reconstituted bursas stained with MGP. (A) Bursal remnant from an 8-week-old chicken that was prepared with cyclophosphamide but did not receive an embryonic bursal transplant. ($\times 30$.) (B) Normal bursal follicles forming early in reconstitution. ($\times 150$.) Note epithelial cores with pyrinophilic lymphoblasts near basement membrane (e) and the developing follicular medulla (m). (C) More advanced follicles show an early cortex (c), basement membrane (bm), capillaries (cap), medulla (m), and follicle-associated epithelium (e). ($\times 150$.) (D) Early reconstitution in bursas transplanted with HB1-infected embryonic bursal cells, showing formation of mixtures of normal and preneoplastic transformed (red staining) follicles. ($\times 30$.) (E and F) Formation of transformed follicles (tf) on epithelial core structures (e). ($\times 150$.) Note initial appearance of pyrinophilic lymphoblasts at the periphery of the epithelial structure with no evidence of differentiation as cells fill the follicle. (G and H) More advanced bursal reconstitution with HB1-infected cells, with about 10% transformed follicles (G; $\times 15$) and with about 70% transformed follicles (H; $\times 30$).

them into chicken embryo recipients whose endogenous bursal cells had been ablated by treatment with the alkylating agent cyclophosphamide.

We selected the myelocytomatosis virus HB1 for this experiment. The transforming gene of this virus appears to have been derived directly from *c-myc* by a recombination event between a transformation-defective deletion mutant of the prototype myelocytomatosis virus MC-29 and endogenous chicken *c-myc* sequences resulting in a hybrid transforming gene (16, 17). Since standard infection of birds with HB1 is reported to induce predominantly bursa-independent lymphomas (18), we reasoned that direct high-multiplicity infection of embryonic bursal stem cells followed by bursal transplantation might be necessary to observe the effect of activated *myc* on bursal lymphocytes. In addition, and in contrast to the experience of Hayward *et al.* (19), preliminary experiments indicated that MC-29 replicated very poorly in bursal lymphocytes in our chicken flocks and induced little or no pathological change in bursas during reconstitution experiments (data not shown).

Induction of Preneoplastic Transformed Follicles with HB1. About 10^6 bursal cells from 18-day embryos were infected with 10^6 to 10^7 infectious HB1 virus particles and transplanted into 18-day embryos that had been treated with cyclophosphamide. Among 117 birds reconstituted with HB1-infected bursal cells, 45 developed tumors. All of these were in the liver, and a majority were hemorrhagic cystic endotheliomas typical of avian myelocytomatosis virus infection. Five birds showed various degrees of liver infiltration with lymphoma (in two cases present simultaneously with an endothelioma). We examined 79 reconstituted bursas in this group for transformed follicles 10–44 days after hatching. During this period bursas showed reconstitution that varied from early follicle formation to fully developed architecture in different individuals (Fig. 1). About 52% of these HB1-infected reconstituted bursas contained more than 1% transformed follicles and 12% of bursas contained more than 50% transformed follicles. This is in marked contrast to experiments with LLV alone, in which no more than 0.2% of bursal follicles are transformed (12). Additionally, no transformed follicles were observed among 10 chickens that received a bursal transplant infected with the specific helper LLV (tdB77) in HB1 stocks (data not shown). Fig. 1 depicts the appearance of follicles in MGP-stained histologic sections of bursas in various stages of reconstitution. In the absence of bursal transplants, the bursal remnant in cyclophosphamide-treated embryos was nearly free of lymphocytes (Fig. 1A) but, as previously described (15), developed various numbers of epithelial core structures bounded by a basement membrane contiguous with that of the gut epithelium and surrounded by a rich network of small capillaries. Examples of these structures are shown in Fig. 1B and E. In reconstitution of normal follicles the first observable event is the appearance of large pyrinophilic lymphoblasts near the basement membrane. As the follicle develops, these cells appear inside the membrane along with the rough gradient of progressively smaller and less pyrinophilic lymphocytes toward the center of the follicular medulla (Fig. 1B and C). The follicle cortex appears last (Fig. 1C). In HB1-infected bursal transplants, we observed the formation of follicles composed solely of lymphoblasts with intensely red staining cytoplasm present with normal follicles in various proportions (Fig. 1D). In the development of these transformed follicles, pyrinophilic lymphoblasts also appear initially along the basement membrane, but then they proliferate and migrate to the medullary and cortical regions of the follicle with no evidence of differentiation (Fig. 1E and F). These large lymphoblasts eventually make up the entire lymphoid population of the transformed follicle. These HB1-induced transformed follicles were also seen in more fully reconstituted

bursas (Fig. 1E and F) and showed the same histological features as the preneoplastic transformed follicles observed after infection with LLV (11, 12). Rarely (less than 5%) transformed follicles showed some evidence of enlargement, disorganization, and local invasion, but gross neoplastic progression was not seen for the 4-week period after hatching during which we were able to make observations.

We note that the oncogenic spectrum of the HB1 virus in these chickens was significantly different than that previously reported for this virus in Brown Leghorn birds (which failed to develop endotheliomas) and inbred line 151 White Leghorns (which failed to develop any HB1-induced tumors) (18). We do not know the basis for these differences. It is also not clear why in our preliminary experiments, and in the study reported by Enrietto *et al.* (18), we did not induce bursal neoplasia with standard MC-29 as reported by Hayward *et al.* (19). It seems reasonable to speculate that the use of different strains of birds and possibly different routes of infection underlie this variability. We also assume that our ability to induce bursal pathology with HB1 when standard infection of birds with this virus does not do so (18) is a function of direct exposure of embryonic bursal stem cells to high-multiplicity infection.

HB1 *v-myc* DNA in Reconstituted Bursas. DNA from five bursas, which were found to contain various proportions of transformed follicles by histologic examination of small samples, was analyzed for the presence of the HB1 *v-myc* gene by restriction endonuclease digestion, agarose gel electrophoresis, and blot hybridization. The results are displayed in Fig. 2. The figure shows partial restriction maps of the *c-myc* locus (20) and the HB1 provirus (16). Digestion with *Bam*HI should generate a 10-kilobase (kb) fragment containing the endogenous *c-myc* locus and a 2.9-kb fragment of the HB1 provirus bearing the *v-myc* gene. By using a 32 P-labeled

FIG. 2. *Bam*HI digestion of *myc* DNA in reconstituted bursas. (A) DNA from HB1-transformed QEF (lane 1), from reconstituted bursas with about 100% (lane 2), 80% (lane 3), 50% (lane 4), and less than 10% transformed follicles (lanes 5 and 6) and from normal chicken bursa (lane 7) was digested with *Bam*HI and analyzed by agarose gel electrophoresis and blot hybridization using an equimolar ratio of *c-myc* exon probes (shown as the E fragments in the restriction map of *c-myc*). (B) The same lanes were washed to remove the E probe and rehybridized with the *c-myc* intron probe (shown as the I fragment in the restriction map in C).

probe composed of equal molar mixtures of plasmids containing cloned *c-myc* exon fragments (the E probes shown in Fig. 2), this diagnostic 2.9-kb *v-myc* fragment was detected in *Bam*HI digests of DNA from HB1-transformed quail embryo fibroblasts and from reconstituted bursas containing high (greater than 50%) and intermediate (20–50%) proportions of transformed follicles. In DNA from bursas containing 10% or less transformed follicles, and in normal bursal DNA, this fragment was not detected. To demonstrate that the 2.9-kb *Bam*HI fragment was not derived in some fashion from a rearranged *c-myc* locus, the blots were washed under conditions that removed the signal from hybridized exon probe and then were reprobed with a labeled plasmid bearing sequences from the intron of the *c-myc* locus (the I probe shown in Fig. 2). Results, shown in Fig. 2B, indicate that, as expected, this probe failed to detect the 2.9-kb *Bam*HI fragment but did hybridize to the 10-kb endogenous *c-myc* band from all of the chicken DNA *Bam*HI digests. We concluded that the HB1 *v-myc* gene was detectable by this technique primarily in bursas containing a moderate to high proportion of preneoplastic transformed follicles.

Two unexpected fragments were also detected in these *Bam*HI digests. A 6.8-kb band (Fig. 2, lanes 2, 3, 5, and 7) was detected with both the exon and intron *c-myc* probes in some DNA preparations. Because this fragment was present in normal bursal DNA in some cases (Fig. 2, lane 7), and contained intron sequences, we do not believe it is related to the HB1 infection. A reasonable explanation is the presence of a *Bam*HI polymorphism near the *c-myc* locus in these birds. Finally, a 3.5-kb fragment was observed in the *Bam*HI digests from one bursa containing about 70% transformed follicles (Fig. 2A, lane 3). Since this fragment lacked intron sequences, we suggest it represents an altered HB1 provirus as has been previously observed in HB1-induced lymphomas (18).

NIH/3T3 Transforming Genes in Reconstituted Bursas. The ability to induce large numbers of transformed follicles by this technique provided us with our first opportunity to assess the presence of activated *Blym*-1, or other similar oncogenes at this preneoplastic stage. Transfection assays were carried out on NIH/3T3 cells with high molecular weight DNA from reconstituted bursas containing transformed follicles. Controls in each experiment included normal chicken or mouse DNA and DNA from the bursal lymphoma cell line RP-9 (from which *Blym*-1 was ultimately derived) or from NIH cells transformed by DNA from a neoplastic bursal nodule [NIH(BN2993)]. The RP-9 DNA was equal in transforming activity to DNA from fresh bursal nodules and lymphomas (4). The results of these experiments are summarized in Table 1. DNAs from 13 reconstituted bursas containing transformed follicles in proportions varying from 5% to 100% failed to induce foci above the background level observed with normal DNA. RP-9 DNA was 8- to 28-fold more active in transformation than DNA from bursas with transformed follicles. To establish the sensitivity of the assay and to exclude an inhibitory effect from the normal bursal DNA in the preparations, RP-9 lymphoma DNA was mixed in various proportions with normal bursal DNA and transfection assays were carried out. Fig. 3 shows the results of these assays compared with transformation by DNA from bursas with preneoplastic transformed follicles carried out in the same experiment. The transforming activity of lymphoma DNA could be distinguished from that of preneoplastic transformed follicle cell DNA even when derived from a mixture in which the transformed cells represented only 10–20% of the total. Therefore, bursal cell DNA containing the HB1 *v-myc* gene failed to transform NIH/3T3 cells. We do not know whether this is a specific property of this particular virus or reflects the previously reported low efficiency of *v-myc* transformation in this assay (21). The

Table 1. Transformation of NIH/3T3 cells

Donor DNA	% transformed follicles or tumor*	Birds	Total foci/total plates [†]	Foci/ μ g of DNA [‡]
Normal§	0	—	13/22	0.03
HB1-infected	1–10	4	6/12	0.025
bursas with	10–20	4	3/18	0.008
transformed	20–50	2	1/5	0.010
follicles	50–100	3	4/7	0.028
Totals		13	14/42	0.016
HB1-infected bursa				
83	15	1	17/8	0.11
Hepatic lymphoma¶	80	1	21/4	0.26
RP-9 lymphoma	100	—	74/16	0.23
Cell line				
NIH(BN2993)	100	—	15/6	0.13

Transformation refers to DNA-mediated focus formation in transfection assays.

*Percent of the tissue or cell line composed of morphologically transformed cells.

[†]Each plate was seeded with 5×10^5 NIH/3T3 cells and transfected with 20 μ g of donor DNA.

[‡]DNA from each reconstituted bursa was assayed separately. Results were pooled in groups with comparable percentages of preneoplastic transformed follicles.

§Normal chicken or mouse DNA.

¶Liver extensively infiltrated with lymphoma in an HB1-infected reconstituted chicken with transformed follicles in bursa.

||NIH/3T3 cells transformed with bursal nodule DNA (3).

significant conclusion is that HB1-induced transformed follicle cells usually lack activated transforming genes such as *Blym*-1.

There was a single observation, shown in Fig. 3 and listed as bursa 83 in Table 1, in which DNA from a bursa with transformed follicles induced significant transformation in NIH/3T3 cells. One interpretation of this exception is that this event reflects progression to a more advanced neoplastic stage in this particular bursa. DNA from a liver extensive-

FIG. 3. Transformation of NIH/3T3 cells by DNA from transformed follicle cells and lymphoma cells. The numbers of transformed foci induced in NIH/3T3 cells by DNA from reconstituted bursas containing various proportions of transformed follicles (●) were compared with the numbers of foci induced by comparable mixtures of normal bursal and RP-9 lymphoma cell line DNA (○).

ly infiltrated with a malignant lymphoma from one of these birds was also positive in the transfection assay (Table 1). Because HB1 is known to induce bursa-independent lymphomas in some chicken strains (18), we do not know whether the lymphomas we detected in this experiment represented metastases from progressing bursal neoplasms or were independent events. If they were independently induced by HB1 it appears that, like advanced bursal lymphomas, they contained activated transforming genes.

DISCUSSION

We have described a bursal transplantation model in which candidate oncogenes can be inserted into stem cells and their effects can be assessed in reconstruction of the organ and the tumor system. The principal conclusion drawn from the initial exploitation of this system is that an activated HB1 *myc* gene can rapidly and efficiently induce the formation of preneoplastic transformed follicles. The cellular target for the activity of this oncogene in the bursa is a large pyrinophilic lymphoblast. On the basis of our observations in this transplantation model, we speculate that normal cells of this type are of donor origin, home to the follicular basement membrane, and act as progenitors for B-cell differentiation at least in the medulla of the bursal follicle. Experimental verification of this proposal, including the use of appropriate markers for donor cells, should be possible in this model. In any case, it is clear that the introduction of a *v-myc* gene into these cells results in their failure to differentiate. Instead, they repopulate the follicle, forming distinctive lesions that are indistinguishable from the preneoplastic transformed follicles that arise at much lower frequency in LLV-induced bursal lymphomagenesis. This observation suggests that the *myc* genes activated either by integration of LLV long terminal repeat (LTR) sequences or by incorporation into the HB1 genome induce similar pathology in bursal lymphocytes.

In the majority of HB1-induced preneoplastic follicles we did not detect biologically active NIH/3T3 transforming genes such as *Blym-1*. We therefore conclude that an activated *myc* gene is alone sufficient to induce the preneoplastic follicle state of lymphomagenesis. Since NIH/3T3 transforming genes have been detected in all neoplastic bursal lymphomas studied (4), these results are consistent with the hypothesis that activation of *Blym-1*, or similar NIH/3T3 transforming genes, is associated with progression of preneoplastic lymphocytes to clonal neoplastic growth. Although detection of NIH/3T3 transforming activity in DNAs of one HB1-infected bursa and a hepatic lymphoma suggest that transforming gene activation can occur during the course of HB1-induced disease, we did not systematically study more advanced bursal neoplasias in this set of experiments. Such studies will be facilitated by experimental strategies that avoid the lethal effects of viral spread to other organs, which limited the observation period to about 4 weeks after hatching in the present study.

Nevertheless, these findings, as far as they go, provide additional support for the idea that the oncogenes associated with LLV-induced bursal lymphomas act in a stage-specific

manner. This hypothesis, simply stated, is that transcriptional activation of *myc* results in the proliferation of a particular type of lymphoblast confined within bursal follicles. Activation of *Blym-1* is a second event in one of these preneoplastic follicle cells required for progression to clonal neoplastic growth. This hypothesis can be tested by using retrovirus vectors to define the effects of *Blym-1*, with and without activated *myc*, on bursal lymphocytes in this system.

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