Comparison of chemically induced and spontaneous murine thymic lymphomas in RF and AKR mice: Differential expression of *c-myc* and *c-myb*

(chemical carcinogenesis/oncogene expression/3-methylcholanthrene)

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ABSTRACT Expression of 11 cellular oncogenes was determined in normal vs. lymphomatous thymic tissues of RF and AKR mice; only *c-myc* and *c-myb* transcripts were detected in an age-inappropriate pattern in thymomas. Normal thymocytes from young RF mice contained RNA transcripts of both genes, but the transcripts were no longer detected at 9 or more weeks of age. More than 90% of RF thymomas, occurring at 20–28 weeks of age after skin painting with 3-methylcholanthrene at 12 weeks, contained *c-myc* transcripts, and 70% of the tumors contained *c-myb* transcripts. Seven cell lines derived from these 3-methylcholanthrene thymomas expressed both cellular genes, as did 2 rare spontaneous thymomas of 12-month-old RF mice. No indication of rearrangement or amplification of either gene was seen in any of the RF tumors or cell lines. In AKR mice, transcripts of the 2 genes persisted longer in the normal thymus than in RF mice, but they were no longer detected at 26 weeks of age. Of 3 thymomas in 6-month-old 3-methylcholanthrene-treated AKR mice, all expressed *c-myb* and 2 expressed *c-myc*. Among 11 spontaneous AKR thymomas, however, only 2 showed detectable levels of both genes, and 2 more expressed *c-myc* or *c-myb* but not both.

After topical application of the polycyclic hydrocarbon 3-methylcholanthrene (MCA) at 3 months of age, the inbred mouse strain RF demonstrates a near 100% incidence of thymic lymphoma within 2–5 months. In contrast, untreated RF mice do not develop thymic lymphomas prior to 10 months of age and only show a 25% incidence of disease between 12 and 24 months of age (1). Therefore, this system provides a specific type of tumor (T-lymphocyte lymphoma) that can be induced by a chemical carcinogen under controlled experimental conditions. Attractions of this model system are that the properties of the tumor cells can be directly compared to those of age-matched normal thymic tissue, and that tumors induced by a chemical carcinogen can be compared to those induced by retroviruses.

The RF mouse strain is closely related to another inbred mouse strain, AKR, both established >50 years ago from a random breeding population. However, almost all AKR mice spontaneously develop thymic lymphoma at 7–12 months of age, in contrast to the <5% disease incidence characteristic of RF mice of equivalent age (2, 3). AKR mice are also susceptible to MCA treatment. Topical application of MCA at 3 months of age to this mouse strain induces a 2-month acceleration in the time of appearance of thymic lymphomas (1). Thus, treatment of both RF and AKR mice with MCA results in the development of thymic lymphoma at 5–8 months of age in nearly 100% of treated mice. The MCA-induced disease is similar at gross autopsy to the spontane-

ous disease observed in AKR or RF mice, and both thymic diseases involve malignant T-cell lymphocytes (4) that often metastasize to the spleen and lymph nodes. Although much evidence implicates recombinant murine leukemia virus (MuLV) as an essential agent in the spontaneous disease of AKR mice (for review, see ref. 5), no such evidence exists for involvement of these viruses in MCA-induced lymphomas (6). MCA-induced primary lymphomas in RF mice only rarely contain novel murine leukemia virus insertions and MuLV RNA expression is generally the same in normal thymocytes and primary tumors.

Normal vertebrate cells contain sequences related to genes carried by acutely transforming retroviruses that encode the ability to induce malignant disease. Examination of malignant tumors from various sources has revealed that these cellular sequences, called cellular or proto-oncogenes, can be altered by translocation, amplification, MuLV insertion, or simple point mutations (for reviews, see refs. 7–10). We have investigated the relative levels of transcripts derived from several oncogenes in RNA derived from primary thymic tumors induced by MCA and normal thymic tissue obtained from untreated mice age-matched for both time of application of the MCA and the time of appearance of MCA-induced tumors. In addition, we assayed spontaneous thymic tumors of AKR mice for these same oncogene transcripts to determine whether there were differences between MCA-induced and spontaneous thymic T-cell lymphomas. We report here that most chemically induced tumors contain age-inappropriate expression of mRNA derived from two cellular oncogenes, *c-myc* and *c-myb*. These same genes appear to be expressed much less frequently in spontaneous tumors of AKR mice.

EXPERIMENTAL PROCEDURES

Mice. RF/J mice were obtained from The Jackson Laboratory. AKR/J mice were obtained from the colony of Maria Duran-Reynals (Albert Einstein College of Medicine).

Treatment with MCA. Female mice of the RF/J inbred strain were treated with MCA at 12–14 weeks of age (1). The backs and flanks of the mice were shaved and then painted daily for 5 days with a 1% (wt/vol) solution of MCA (Tridom Chemical, Hauppauge, NY) in benzene. Control animals were either treated with benzene alone or were untreated. Animals with thymic lymphoma were identified by their labored breathing and palpable lymph nodes.

DNA and RNA Extraction and Hybridization. DNA was extracted from both tissues and cultured cells as described

Abbreviations: MCA, 3-methylcholanthrene; MuLV, murine leukemia virus; kb, kilobase(s).

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(11). After digestion with restriction enzymes (New England Biolabs) under the conditions recommended by the supplier, DNA was fractionated by electrophoresis on 0.6% agarose (SeaKem Laboratories, Rockland, ME) at 50 V for 16–20 hr (1.6 V/cm) and transferred to nitrocellulose (Schleicher & Schuell) (12).

Total cellular RNA from both tissues and cell lines was prepared by a modified guanidine-HCl method (13). RNA was denatured in 65% (vol/vol) formamide/6% (vol/vol) formaldehyde at 65°C, fractionated by electrophoresis on 1% agarose gels containing 3% formaldehyde in 200 mM 3-(*N*-morpholino)propanesulfonic acid buffer (pH 7.5) with 0.001 M EDTA, and transferred to Gene Screen (New England Nuclear). Hybridizations with *c-myc* DNA probes were carried out for 18 hr in 5× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate)/50% formamide/5× Denhardt's solution (14)/0.01 M phosphate buffer, pH 6.5/100 µg of denatured salmon sperm DNA per ml/10% (wt/vol) dextran sulfate at 37°C. Hybridizations with *v-myb* DNA were done identically except that 40% formamide was used.

Hybridization Probes. DNA hybridization probes specific for *c-myc* and *v-myb* were prepared by nick-translation (15) of isolated restriction endonuclease-generated DNA fragments. The murine *c-myc* DNA probe (pMc-myc 54), was kindly provided by Rosemary Watt and Kenneth Marcu (16). Regions of the cloned DNA specific for exons 1, 2, and 3 were prepared by *Pst* I digestion, and isolation of the appropriately sized fragments was done on agarose gels. The DNA probe for exon 1 of *c-myc* and the 5' cellular flanking sequence was kindly provided by Laurie Eckhardt, Barbara Birshstein, and Kenneth Marcu (see ref. 17; *Eco*RI/*Eco*RI* fragment subcloned into pBR325). Marcel A. Baluda kindly supplied the chicken *v-myb* probe (pBR322/HAX 4) (18). The *myb*-specific fragment was prepared from this fragment by digestion with *Bam*HI. The plasmid clones for ribosomal protein L1 (pL10; 19), the murine 8B sequence (20), and β_2 -microglobulin were generously provided by A. Skoultchi, L. Leinwand, and T. V. Rajan, respectively; these probes were used as standards for determining the quality of RNA.

RESULTS

RF Normal Thymus and Thymomas. DNA probes for 11 cellular oncogenes were used to detect RNA species in cells of 10 individual MCA-induced RF primary tumors, tumor-derived cell lines, and normal thymic tissue. RNA species related to the following *c-onc* genes were investigated: *abl*, *mos*, *fes*, *rel*, *ras^H*, *ras^K*, *ras^N*, *sis*, *erb^B*, *myc*, *myb*. Only RNA species related to *ras^H*, *ras^N*, *myc*, and *myb* were detected. *ras*-related RNA species were expressed in both normal thymocytes and tumor cells at essentially the same low levels (data not shown). In contrast, the levels of both *myc* and *myb* RNA species were variable compared with normal thymus tissue.

To determine whether the steady-state levels of transcripts found in tumor cells are representative of normal cells found in the thymus, we examined RNA from multiple independent samples of normal thymus from RF mice of different ages. Transcripts of both the *c-myc* and *c-myb* genes were readily detectable in preparations from RF mice 7 weeks old or younger, but neither transcript was detected in samples taken at 9–28 weeks of age (Fig. 1). All RNA gels were hybridized with DNA probes for other cellular sequences [ribosomal protein L1 (19) and murine 8B sequence (20)] to ensure the quality of the samples (data not shown). It thus appears that *c-myc* and *c-myb* are no longer expressed in the normal thymus of RF mice at either the age of MCA treatment (12 weeks) or that of the appearance of MCA-induced thymomas (20–28 weeks).

RNA from 10 primary MCA-induced thymomas were examined for *c-myc* and *c-myb* expression; all 10 tumors contained *c-myc* transcripts, and 7 of them contained *c-myb* transcripts (Fig. 1, lanes 7–16). The variations in levels of these RNAs among the tumors were approximately parallel to those of RNA related to other cellular genes expressed in all cell types (data not shown). Comparison of these results with those from normal thymus indicates that these genes continued to be expressed or are reexpressed in the chemically induced tumors at an age when most of the normal thymocytes found in that tissue would no longer do so. The levels of the *c-myb* transcripts in the tumor tissue were similar to those found in normal thymic tissue of 6-week-old mice, while *c-myc* transcripts in tumor tissue were at least as abundant as those found in 6-week-old thymus and as much as 5-fold higher in some tumors.

Cells of seven cultured lines derived from MCA-induced RF thymomas were examined, because they represent a source enriched for tumor cells. These lines were all tumorigenic *in vivo* and expressed the same pattern of T-cell-specific antigens as the primary thymomas from which they were derived (4). All seven lines contained *c-myc* and *c-myb* RNA at levels equal to or greater than those in 7-week-old RF mice (Fig. 2). Thus, despite having been in continuous culture for 6–24 months since explantation, the cell lines were similar to primary tumors in expression of these two genes.

Spontaneous thymomas occur with a low incidence in RF mice but rarely before 12 months of age. We were able to examine only two of these tumors that occurred in 10- to 12-month-old mice. Both tumors contained *c-myc* and *c-myb* transcripts at levels similar to those in MCA-induced lymphomas (see Fig. 4). The transcripts expressed in all tumor cells examined were indistinguishable in size from their normal cell counterparts and, in the case of *c-myc*, contained all three transcribed exons (data not shown).

AKR Normal Thymus and Thymomas. Normal AKR thymic tissue expressed *c-myc* and *c-myb* transcripts until the age of 20 weeks, markedly later in life than RF mice. However, at 26 weeks and later these transcripts were no longer detected (Fig. 3). RF mice may be unusual among inbred strains in their much earlier shutdown of *c-myb* in the thymus, because *c-myb* transcripts can also be detected in 16-

FIG. 1. Detection of *c-myc* and *c-myb* transcripts in RNA from thymic tissue of normal RF mice and MCA-induced thymic lymphomas. Total cellular RNA (20 µg) obtained from thymic tissue of normal RF mice 6 weeks, 9 weeks, 12 weeks, 6 months, and 6.5 months old (lanes 1–5) and from thymic lymphomas present in 6- to 7-month-old RF mice previously treated with MCA at 3 months (12–14 weeks) of age (lanes 6–15) were analyzed for *c-myc* (Upper) and *c-myb* (Lower) transcripts.

week-old BALB/c thymic tissue (21). BALB/c mice older than 16 weeks were not examined for age-related changes in *c-myb* expression (21).

Spontaneous thymomas from 11 untreated AKR mice aged 7–10 months were examined for *c-myc* and *c-myb* transcripts (Fig. 4). Among these tumors, 7 showed no detectable levels of transcripts from either gene, 2 expressed both genes (AKR-1, AKR-14), and 2 expressed either *c-myc* (AKR-10) or *c-myb* (AKR-2) transcripts but not both. The presence of these transcripts in only a relatively small percentage of AKR spontaneous thymomas is in marked contrast to their presence in almost all RF MCA-induced thymomas. The apparent difference in the expression of these two genes in spontaneous AKR tumors and in MCA-induced tumors is not due to the quality of the RNA samples. All AKR RNA samples had intact MuLV genomic RNA (6). Also, tumor AKR-2 expressed similar amounts of β_2 -microglobulin mRNA as RF tumor (no. 15) but contains only 30% as much *c-myb* mRNA and no detectable *c-myc* mRNA (Fig. 5).

Of three thymomas occurring at 6 months of age in AKR mice treated with MCA at 3 months, all three showed significant levels of *c-myb* transcripts, and two showed readily detectable levels of *c-myc* transcripts (Fig. 3). Since these lymphomas occurred earlier than the spontaneous lymphomas of this strain, they are likely to be the result of MCA treatment. Although the number of tumors examined was small, these findings suggest that AKR spontaneous thymomas differ from RF spontaneous thymomas and from MCA-induced thymomas of both RF and AKR mice in their low-frequency expression of the *c-myc* and *c-myb* genes.

Analysis of *c-myc* and *c-myb* DNA in Thymomas and Normal Thymus. Although the size of *c-myc* and *c-myb* transcripts was identical in all samples of both thymomas and normal thymus, we also examined preparations of DNA treated with restriction enzymes for evidence of amplification or rearrangement of these genes in the tumor cells (Figs. 6 and 7). In no case was evidence for alterations in these genomic regions observed. For the *c-myc* gene, our analysis extends 8 kilobases (kb) upstream of the gene and 7 kb 3' to exon 3.

FIG. 2. Detection of *c-myc* and *c-myb* transcripts in the RNA from seven independent MCA-induced RF thymic lymphoma cell lines. Poly(A)-enriched RNA (4 μ g) prepared from passaged MCA-induced RF lymphoma cell line MC412 and total cellular RNA (20 μ g) from seven independent MCA-induced RF lymphoma cell lines passaged for 6 months to 1 year *in vitro* were analyzed for *c-myc*, *c-myb*, and murine CHO-b (8B) transcripts. A single RNA blot was used for this analysis: after hybridization and autoradiographic exposure, the probe was removed, and the same blot was subsequently hybridized with the indicated probe. Only a single hybridized RNA species was observed in each case and is indicated on the right. Positions of 28S and 18S rRNA are indicated on the left.

FIG. 3. Detection of *c-myb* and *c-myc* RNA species in normal thymic tissue of aging AKR mice and in thymic lymphomas of MCA-treated AKR mice. Total cellular RNA (20 μ g) prepared from thymic tissue of AKR mice ages 4 weeks, 9 weeks, 14 weeks, 20 weeks, and 26 weeks (6 months) as well as from thymic lymphomas obtained from 6-month-old AKR mice previously treated at 12 weeks of age with MCA (lanes 10–12) were analyzed for *c-myb* and *c-myc* transcripts as described. Parallel samples were used for the hybridization analysis with the *myb* (Upper) and *myc* (Lower) DNA probes. Lanes 1–9 are from a single blot and lanes 10–13 are from a separate blot. Two of the three 20-week samples were from unenlarged AKR thymic tissue (lanes 5 and 6). The third 20-week sample (lane 7) was from a clearly enlarged thymic organ considered to be a young tumor. The two 26-week samples (lanes 8 and 9) and the 6-month AKR sample (lane 13) were from unenlarged thymic organs.

The murine *c-myb* gene has not been characterized, so that our analysis must be limited to those fragments homologous with the chicken *v-myb* probe used. These results are consistent with the absence of acquired MuLV genomes in MCA-induced thymomas as well as the apparently normal karyotype of RF MCA cell lines (unpublished observations).

FIG. 4. *c-myc* and *c-myb* expression in spontaneous AKR and RF thymic lymphomas. Total cellular RNA (20 μ g) from several independent spontaneous AKR thymic lymphomas from mice 7–10 months old and two independent RF thymic lymphomas from untreated 10-month-old RF mice were analyzed for the presence of *c-myc* and *c-myb* RNA species as described. A positive control sample (thymic tissue RNA from 9-week-old AKR) was included in each hybridization (Upper, lanes 1 and 14; Lower, lanes 1 and 13). Only a single hybridizing RNA species was detected with either probe and only these areas of the autoradiogram are shown. Positions of 18S and 28S rRNA are indicated.

FIG. 5. Differences in expression of *c-myc* between normal and lymphomatous tissues expressing *c-myb*. Poly(A)-enriched RNA (5 μ g) from normal thymic tissue of 7 week RF mice (lane 1), MCA-induced RF thymic lymphoma (sample 15; lane 2), and spontaneous AKR thymic lymphoma (sample AKR-2; lane 3) are shown hybridized to DNA probes for *myc*, *myb*, and β_2 -microglobulin on the same blot. Samples were first hybridized to *myb* DNA and the blot was subsequently erased and rehybridized to the other DNA probes.

DISCUSSION

Recent studies in our laboratories have produced several lines of evidence suggesting that thymic lymphomas induced in RF mice by skin painting with MCA differ significantly from spontaneous thymic lymphomas of AKR mice, although the two diseases are indistinguishable at gross autopsy. Whereas most spontaneous AKR thymomas express high levels of *Thy-1* and low levels of *H-2K/H-2D* antigens on their surfaces (21) (a phenotype resembling that of cortical thymocytes), MCA-induced RF thymomas show the reverse pattern: high *H-2K/H-2D* and low *Thy-1* antigens (4) (a phenotype more characteristic of medullary thymocytes). Morphologic studies of the prelymphomatous thymus in the two systems show, perhaps paradoxically, that the spontaneous thymomas arise in the thymic medulla, whereas the MCA-induced thymomas arise in the cortex (unpublished results). Although many studies have demonstrated an important role for endogenous MuLV in spontaneous AKR thymomas, studies to date of MCA-induced RF thymomas have produced no evidence for an etiological role of these viruses (6).

We have now compared the steady-state levels of two proto-oncogene transcripts, *c-myc* and *c-myb*, in MCA-induced RF thymomas and spontaneous AKR thymomas. Among the chemically induced tumors, almost all expressed *c-myc* mRNA, and most expressed *c-myb* RNA. By contrast, only 4 of 11 spontaneous AKR thymomas expressed one or both of these two genes. Normal mice of these strains were found to cease producing detectable levels of transcripts of these genes in the thymus at an earlier age than that of the appearance of the tumors and, in the case of RF mice, that of MCA treatment. We conclude that the T lymphocytes comprising tumors induced by MCA differ from the T lymphocytes in spontaneous AKR tumors in the frequency with which they express *c-myc* and *c-myb* transcripts. Considering the differences in differentiation antigen expression, MuLV expression, and now oncogene expression in these lymphoma cells, it seems clear that they are distinct diseases.

In tumors in which cellular oncogenes have been observed to be amplified, translocated, or interrupted by insertion of MuLV proviral sequences (16, 23–30), it is reasonable to ar-

gue that the affected gene has an etiological role in the initiation or maintenance of the transformed phenotype. MCF-like viral DNA sequences integrate near *c-myc* in $\approx 25\%$ of virally induced or spontaneous T-cell lymphomas (29). In these tumor cells, the proximity of the viral long terminal repeat enhancer to *c-myc* presumably allows persistent expression of *c-myc* transcripts. Although we have not examined the *c-myc* locus of spontaneous AKR lymphomas, the frequency that *c-myc* transcripts are detectable (3 of 11 tumors) closely parallels the frequency of viral integration observed by Corcoran *et al.* (29). The identification of a consistent alteration of the *c-myc* locus indicates that this gene likely contributes to the genesis of the tumor. The *c-myb* locus has not been examined in a similar fashion in AKR lymphomas; however, the high frequency of acquired proviral sequences in tumor-cell DNA (31) may warrant further investigation of this possibility.

In contrast, the absence of these gross alterations of *c-myc* and *c-myb* in MCA-induced tumors (Figs. 6 and 7) makes it more difficult to argue for a similar role of these genes in MCA-induced lymphomagenesis in RF mice. It remains possible that there are subtle alterations in these genes not detectable by our restriction endonuclease analysis. Studies on the *ras* gene family have demonstrated that a single amino acid change is sufficient to result in an allele capable of transforming NIH 3T3 fibroblasts (32). Somatic mutation of the human *c-myc* gene has been postulated as a contributing factor in the altered regulation of *c-myc* seen in several Burkitt lymphoma cell lines (33, 34). Mutations in the *c-myc* and *c-myb* genes or in unlinked regulatory genes may, therefore, be responsible for the persistent expression of mRNA seen in tumor samples. The increased expression of *c-myc* mRNA observed in a large number of human hematopoietic leukemias and lymphomas is in most instances also not due to obvious alterations in the *c-myc* gene (35).

The expression of the normal *c-myc* allele is induced by growth factors in a cell-cycle-dependent manner (36). However, in chemically transformed fibroblasts, *c-myc* loses its

FIG. 6. Absence of *c-myb* rearrangement or amplification in MCA-induced RF lymphoma cell lines. Genomic DNA (12 μ g) isolated from 12-week-old RF thymus (lanes 1, 5, and 9) and three MCA-induced RF lymphoma cell lines (MC 412, MC 409, MC 106) was digested with *Bam*HI, *Hind*III, or *Eco*RI, separated by electrophoresis on 0.6% agarose, transferred to nitrocellulose, and hybridized with 32 P-labeled *v-myb* DNA (HAX 4 as described in *Experimental Procedures*). Sizes (kb) of the λ *Hind*III marker DNA are indicated on the left.

FIG. 7. Absence of *c-myc* DNA rearrangement or amplification in MCA-induced RF tumors. Genomic DNA (12 μ g) isolated from the liver and thymus of 12-week-old RF mice, MCA-induced RF thymic lymphomas taken from 6- to 7-month-old RF mice, and two spontaneous RF thymic lymphomas appearing in 10-month-old RF mice was digested with *Eco*RI (A) or *Bam*HI, resolved by electrophoresis on 0.6% agarose, transferred to nitrocellulose, and hybridized with 32 P-labeled pmc54 DNA. Sizes of λ *Hind*III fragment markers are indicated. (A) Typical example of several independent experiments that clearly indicated the lack of genomic rearrangements in four MCA-induced RF primary tumors (lanes 3-6) and two spontaneous RF thymic tumors (lanes 7 and 8). (B) Example of several blots that demonstrated no DNA sequence amplification or rearrangement in primary MCA-induced thymic lymphomas (lanes 2 and 3), lymphomas passaged *in vivo* in RF mice (lanes 4-7), or lymphomas passaged as cell lines *in vitro* (lanes 8 and 9). Arrows indicate *c-myc* hybridizing fragments.

cell-cycle-specific regulation (37). Examination of *c-myc* expression in our lymphoma cell lines in response to T-cell growth factors and during the cell cycle may clarify whether this gene is regulated differently in MCA-induced lymphomagenesis relative to normal T-lymphocytes.

The thymus, like most tissues, is composed of a heterogeneous group of cells with different phenotypes. The cells are mainly immature cortical thymocytes, $\approx 15\%$ mature medullary thymocytes, and a small minority of cells with other phenotypes. These ratios may change during normal aging and raise the question of whether they provide an appropriate standard for the cell lineage represented by the tumor cells. It is possible that, rather than being "activated" directly by the carcinogen, MCA acts to transform a specific lymphoid target cell that already expresses high levels of *c-myc* and *c-myb*. The phenotype of MCA-induced lymphoma cells, however, resembles mature medullary thymocytes (4) that contain much less *c-myb* mRNA than their less mature cortical counterparts (21). If the tumor cells are in fact de-

rived from medullary thymocytes, then we can argue that expression of *c-myc* and *c-myb* at inappropriate levels in mature medullary thymocytes does not result in any phenotypic alteration in their stage of differentiation but contributes to their loss of growth control.

Many results support the idea that oncogenic transformation is a multistep process that may require the cooperation of two or more altered oncogenes (22). It remains to be determined whether *c-myc* and *c-myb* exert such cooperative effects when their expression is altered in target lymphoid cells. In addition, as yet unidentified cellular functions may be involved in the transformation of T lymphocytes in cooperation with *c-myc* and/or *c-myb*.

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