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Activation of c-myb is an early bone-marrow event in a murine model for acute promonocytic leukemia

(protooncogene/preleukemia/inflammation/reverse transcription-PCR)

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ABSTRACT **Insertional mutagenesis of c-*myb* by Moloney murine leukemia virus occurs in 100% of promonocytic leukemias (MMLS) induced by the virus. These leukemias, which resemble acute monocytic leukemia-M5 in humans are induced only in mice undergoing a peritoneal chronic inflammatory response. We have found that two leukemia-specific gag–myb mRNAs in MML provide molecular markers for detection of preleukemic cells in hematopoietic tissue *in vivo*. The two aberrant RNAs result from splicing of *gag* to either exon 3 or 4 of c-*myb*, depending on the site of proviral integration. After reverse transcription–PCR with nested primers and hybridization with specific gag–myb junction probes, one cell, having aberrant c-myb message, could be detected in a minimum of 10^5 liver cells or 10^6 spleen or bone-marrow cells. This approach was used to examine hematopoietic tissues of mice after pristane injection to induce inflammation and virus inoculation. Cells with gag–myb mRNAs could be detected as early as 2 weeks after virus inoculation. In mice receiving both pristane and virus, there was evidence of preleukemic cells in 83% of the mice by 3 weeks after virus infection. Furthermore, 100% of the mice were positive for preleukemic cells by 8 weeks, even though only 50% of mice have been shown to succumb to MML (peak time for disease latency is 12–16 weeks). Cells with these aberrant c-myb messages were initially detected in the bone marrow, but during intermediate stages of disease development these cells disseminated to the spleen, liver, and granuloma. At preleukemic times, from 3 to 8 weeks after virus infection, a lower percentage of mice were positive in the group that did not receive pristane compared with mice in the group receiving pristane. However, at 18 weeks, 100% of the mice in the group receiving virus only had evidence of cells expressing gag–myb RNA in their spleens and/or bone marrow; it is of interest that mice inoculated with virus alone never develop MML. This approach for detecting preleukemic cells will now allow the study of mechanisms by which these preleukemic cells progress to a more transformed state and, perhaps, to a more differentiated state.**

Acute promonocytic leukemia resembling morphological and biochemically acute monocytic leukemia-M5 in humans can be induced in inbred strains of mice by the i.v. inoculation of murine leukemia virus into mice undergoing a chronic inflammatory response (1–6). In 100% of Moloney murine leukemia virus (Mo-MuLV)-induced promonocytic leukemias (MMLs), rearrangement at the c-*myb* locus occurs as a consequence of virus integration (1). c-*myb* is a protooncogene involved in regulating both cell growth and differentiation (7, 8). This protooncogene has specific DNA-binding activity and has been shown to be a transcriptional activator (7, 8).

Our data indicate that development of this disease may depend not only upon the environment of the peritoneal cavity where the acute cellular expansion occurs but may also depend upon hematopoietic organs. This relationship is suggested by the fact that i.v., but not i.p., inoculation of the virus is effective in causing promonocytic leukemias (2). In addition, removal of the spleen can reduce the incidence of this disease (4). It has been proposed that target cells for transformation may reside in the spleen or that partially transformed cells pass through the spleen as part of the leukemogenic process.

For the present investigation we set out to determine whether insertional mutagenesis of c-*myb* is an early event in the development of MML and determine the location of cells having undergone this activation. We took advantage of the fact that in cells having undergone insertional mutagenesis of the c-*myb* locus by Mo-MuLV there are aberrant leukemia-specific myb RNAs. These RNAs are formed as a result of the splicing of viral *gag* to either exon 3 or 4 of c-*myb*, depending on the site of proviral integration. By using reverse transcription (RT) and two rounds of amplification by the PCR with nested primers, low numbers of cells that express these leukemia-specific RNAs could be detected *in vivo*. Our data show that preleukemic cells with activated c-*myb* are present in the bone marrow as early as 2 to 3 weeks after virus infection. Furthermore, these cells can be detected in spleens, livers, and granulomas of mice at 8 weeks, suggesting a migration from the bone marrow with disease progression. All mice harbor cells with activated c-*myb*, even though only 50% on average are destined to develop full-blown disease and die.

MATERIALS AND METHODS

Virus and Cells. The preparation of NIH 3T3 cells producing Mo-MuLV has been described (2). Stocks of virus for injection were collected as 24-hr supernatants and were titered by the UV-XC plaque assay (2, 9). Promonocytic leukemic cell lines (MML) 2B-4-1, 2B-4-2, 2B-4-7, 2B-4-9, and 2B-4-10 were established as described (4) and maintained in DMEM/10% fetal calf serum (GIBCO/BRL). The mouse pre-B cell line 70Z/3B has been described (10).

Animal Experiments. Female BALB/cAnPt mice were bred and maintained at Hazelton Laboratories (National Cancer Institute contract N01-CB-71085). Some received i.p. injections of 0.5 ml of pristane (Aldrich) at 4–6 weeks of age, and all received i.v. inoculations of 0.5 ml of Mo-MuLV (>10^6 plaque-forming units/ml) 3 weeks after receiving pris-

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Abbreviations: MML, murine leukemia virus-induced promonocytic leukemia; Mo-MuLV, Moloney murine leukemia virus; RT-PCR, reverse transcription–PCR.

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tane or at 7–9 weeks of age. The onset of late-stage MML disease was monitored by preparing smears of ascites cells and staining them with Diff-Quik (American Scientific Products, McGaw Park, IL).

Preparation of Total RNA. Total RNA was prepared by the method of Chomczynski and Sacchi (11). Spleen, liver, bone marrow (two tibiae and two femora), and granuloma were each resuspended in guanidinium thiocyanate extraction buffer as whole organs and then divided into 0.5-ml aliquots for further preparation of RNA. Peritoneal lavage cells were collected in 3 ml of Dulbecco's modified Eagle's medium that was injected into and aspirated from the peritoneal cavity.

RT and Amplification of RNA (RT-PCR). RT of RNA into cDNA and amplification were done according to modified procedures of Kawasaki *et al.* (12) and Ferre and Garduno (13). Briefly, 10 μg of RNA in a 5-μl vol was mixed with 1 μl containing 20 pmol of the 3′ negative-strand primer (PCR primer 1B) (see below) and incubated at 70°C for 10 min. This mixture was then incorporated into a total reaction mixture of 20 μl containing 200 units of Mo-MuLV reverse transcriptase (GIBCO/BRL), 20 units of RNasin (Promega), 0.01 M dithiothreitol, and 1 mM of each dNTP. After incubation at 37°C for 45 min, the mixture was diluted to 100 μl, and additional reagents were added to give final concentrations of 1× PCR buffer (Perkin–Elmer), 1 μM of each primer (1A and 1B), and 0.5 unit of *Taq* polymerase (Perkin–Elmer). A second amplification using nested primers was done in 100 μl by using 5 μl of the first reaction; final concentrations were 1 μM of primers 2A and 2B, 100 μM of each dNTP, and 2.5 units of *Taq* polymerase. Amplification reaction mixtures were overlayered with mineral oil and subjected to thermal cycling in a programmable thermal cycler (MJ Research, Cambridge, MA). The program included an initial 94°C incubation for 30 sec, followed by a three-step cycle that was repeated 25 times and consisted of a 94°C denaturation step for 1 min, a 55°C annealing step for 1 min, and a 72°C elongation step for 1 min. A final incubation was at 72°C for 10 min. Every set of PCR reactions included a negative control that had no template added.

Southern Blot Analysis. Twenty microliters from each PCR reaction was electrophoresed on a 4% gel [3% NuSieve (FMC) and 1% agarose (GIBCO/BRL)]. Gels were blotted and hybridized by using standard techniques (14). Oligonucleotides JEX-3 and JEX-4 were 5′ end labeled and used separately to probe blots.

Synthetic Oligonucleotides. Oligonucleotides used for amplification and sequencing were prepared on an Applied Biosystems DNA synthesizer (model 381A). All sequences are shown 5′ → 3′: PCR primer 1A, GGACTCTGCTGACCGG gag sense (nt 1404–1419) (15); PCR primer 1B, CAGACCAACGCTTCGGACC myb antisense (nt 595–613) (16); PCR primer 2A, CCAATGAAGTCGATGCCGC gag sense (nt 1534–1552) (15); PCR primer 2B, GATGAGTTCAGGGTTCAGCAC myb antisense (nt 517–537) (16); JEX-3 probe, GCTTCTCATCCTGCCTGGG antisense gag–myb (ref. 15, nt 1587–1596; ref. 16, nt 406–416); JEX-4 probe, TGTCCGGTTCTGCCTGGG antisense gag–myb (ref. 15, nt 1587–1596; ref. 16, nt 478–486).

RESULTS

gag–myb RNA in MML Cell Lines. All MML cells tested to date express one of two gag–myb fusion messages, depending on the integration site of Mo-MuLV. These fusion RNAs can be detected in MML cell lines by RT of total RNA and one round of amplification, as shown in Fig. 1. With the same primers—that is, 1A from gag and 1B from myb, two different products can be detected, a 293-bp product for cells with proviral integrated upstream of exon 4 and a 365-bp product for cells that have provirus integrated upstream of exon 3. It

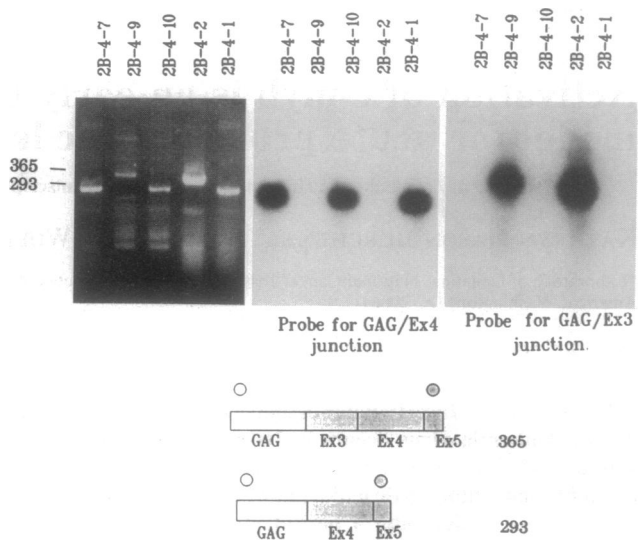

FIG. 1. RT-PCR products showing the expression of gag–myb RNA in MML cells. Total RNA from MML cells was reverse transcribed and subjected to one round of amplification. (*Upper left*) Ethidium bromide-stained, 4% agarose gel. (*Upper middle*) Blot hybridized with JEX-4 probe. (*Upper right*) Blot hybridized with JEX-3 probe. Numbers above panels designate cell lines. (*Lower*) The structure of expected DNA products; circles represent primers 1A and 1B. Ex3, Ex4, and Ex5 refer to c-*myb* exons.

is possible to specifically identify each product by using specific gag–myb junction probes (JEX-3 or JEX-4) as shown in Fig. 1 *Middle* and *Right*. We have found that MML cells that are positive for the *gag*–ex4 junction occur at least twice as frequently as those with the *gag*–ex3 junction (data not shown).

Because we were interested in detecting cells *in vivo* that express gag–myb RNA we wanted to determine the sensitivity level of the RT-PCR assay. Cells from bone marrow, liver, or spleen were mixed with diminished numbers of MML 2B-4-7 cells, and RT-PCR was done on these preparations. With one amplification round the detection level was 1 cell out of 10^4; however, this level was not sensitive enough to detect preleukemic cells *in vivo*. We, therefore, set up the RT-PCR to include two rounds of amplification with nested primers. Fig. 2 shows that the MML cells were detected when diluted with liver cells at a ratio of 1 per 10^5 and detected in bone-marrow and spleen cells at a ratio of 1 per 10^6. The RNAs prepared from the positive 1 per 10^6 samples were

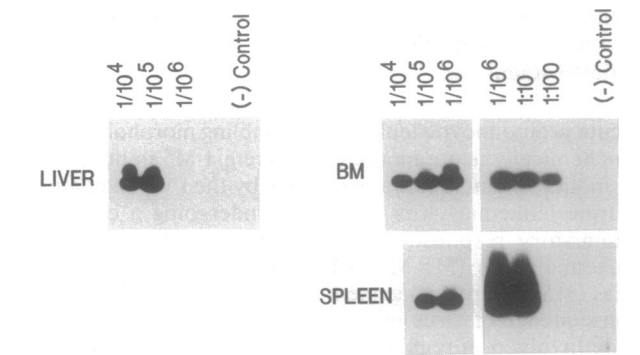

FIG. 2. Sensitivity of RT-PCR approach for detecting cells *in vivo*. Diminished numbers of 2B-4-7 cells were mixed with normal liver, bone marrow (BM), or spleen cells at ratios shown ($1/10^4$–$1/10^6$). RNA was extracted, reverse transcribed, and amplified by two rounds of PCR with nested primers (1A,1B and 2A,2B). Some RNA was further diluted 1:10 or 1:100 with RNA from normal cells and subjected to RT-PCR. Hybridization was done by using JEX-4 probe.

Table 1. Number of mice positive for gag-myb message, as detected by RT-PCR

Weeks after virus	No. positive mice/no. analyzed (%)	
	Virus only	Virus + pristane
1	0 /6 (0)	0 /6 (0)
2	3 /6 (50)	0 /6 (0)
3	2 /6 (33)	5 /6 (83)
5	ND	5 /6 (83)
8	2 /4 (50)	6/6 (100)
18	4/4 (100)	ND

Number of mice positive for gag-myb RNA in one or more tissues with either the JEX-3 or JEX-4 probe. A 10-μg sample of RNA from each tissue (bone marrow, liver, spleen, and granuloma) was subjected to RT-PCR, as described. ND, Not determined.

further diluted 1:10 with RNA from normal bone-marrow and spleen cells and were still positive.

gag–myb RNA in Mouse Tissues during the Preleukemic Phase of MML. To determine when and where activation of the c-*myb* locus occurs during the preleukemic phase of MML disease, hematopoietic tissues of BALB/c mice, inoculated with both pristane and virus, were examined for gag–myb RNA by RT-PCR. At 1, 2, 3, 5, 8, and 18 weeks after virus infection, bone-marrow, spleen, liver, and granuloma cells from four to six mice (all but two groups had six mice) were removed, and RNA was extracted. Cells from peritoneal lavage were collected at 5 and 8 weeks. Ten micrograms of each RNA sample was subjected to RT and two rounds of amplification. Table 1, second column, presents the overall number of mice found positive for the gag–myb RNA in one or more tissues, as detected with either JEX-3 or JEX-4 probes. Eighty-three percent of mice receiving pristane and virus were positive at 3 weeks. Interestingly, as shown in Fig. 3, the gag–myb RNA was found predominantly in the bone marrow. Fig. 3 shows PCR products detected by using probe JEX-4; similar results were obtained with probe JEX-3.

By 8 weeks after virus infection 100% of the mice were positive. As shown in Table 2 and Fig. 4, during the 5- to 8-week preleukemic phase the gag–myb RNA became more

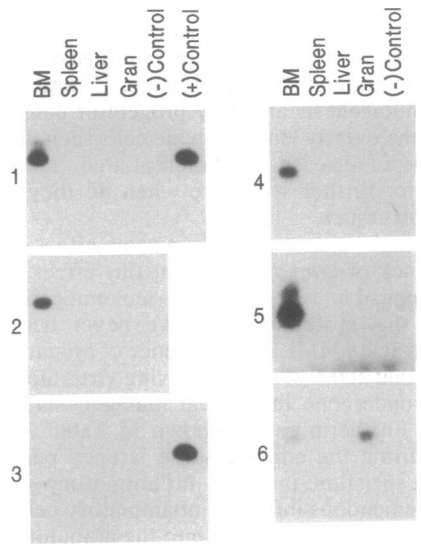

FIG. 3. gag–myb RNA in tissues of pristane- and virus-treated mice 3 weeks after virus infection. RNA extracted from tissues of mice was reverse transcribed and subjected to two rounds of amplification. Positive RNA control was from 703B/3 cells and MML 2B-4-7 cells mixed at a ratio of $1/10^5$. Negative controls had no RNA. Blots were hybridized with JEX-4 probe. (*1–6*) Animals 1–6. BM, bone marrow; Gran, granuloma.

Table 2. Distribution of cells with gag-myb message in tissues of pristane- and Mo-MuLV-injected mice

Weeks after virus	Tissue	No. of mice positive/no. of mice analyzed	
		Gag/ex3*	Gag/ex4†
3	Bone marrow	3/6	5/6
	Spleen	0	0
	Liver	0	0
	Granuloma	0	1/6
5	Bone	1/6	3/6
	Spleen	2/6	0
	Liver	1/6	0
	Granuloma	3/6	1/6
	Peritoneal lavage	3/6	2/6
8	Bone marrow	0	5/6
	Spleen	1/6	3/6
	Liver	0	3/6
	Granuloma	0	3/6
	Peritoneal lavage	0	4/6

Mice were injected with pristane and 3 weeks later inoculated i.v. with Mo-MuLV. Ten micrograms of RNA extracted from tissues was subjected to RT-PCR.
*Mice positive with JEX-3 probe.
†Mice positive with JEX-4 probe.

evident in other organs, including spleen, liver, and granuloma. One mouse, no. 5, had cells expressing the aberrant RNA in every tissue. The PCR products could be detected in the tissues of mouse no. 5 by using only one amplification round. This mouse and one mouse from the 5-week time point were the only examples in which the PCR product could be detected in the absence of a second amplification round.

Mice were found positive for at least two clonal *myb* activation events because at 3, 5, and 8 weeks there were three, four, and one mice, respectively, that had PCR products positive with JEX-3 probe, as well as products positive with JEX-4 probe. This result suggests that this insertional mutagenic event can be biclonal or, perhaps, even multiclonal.

gag–myb RNA in Mice Receiving Virus but No Pristane. Because pristane is required for disease induction, it was of interest to determine its effect on activation of c-*myb* by the

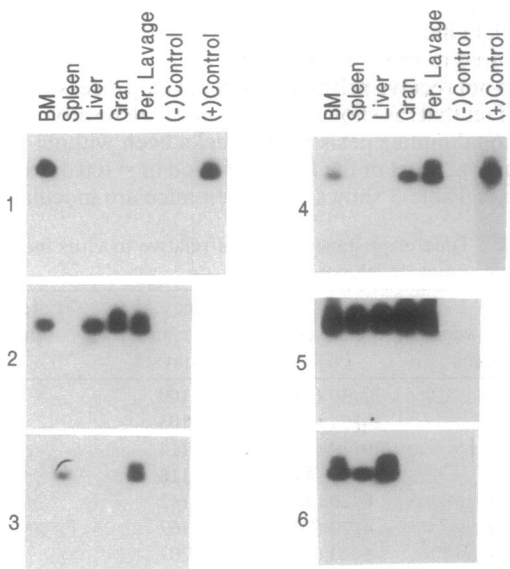

FIG. 4. gag–myb RNA in tissues of pristane- and virus-treated mice 8 weeks after virus infection. Conditions were the same as for Fig. 3. (*1–6*) Animals 1–6. BM, bone marrow; Gran, granuloma; Per. Lavage, peritoneal lavage.

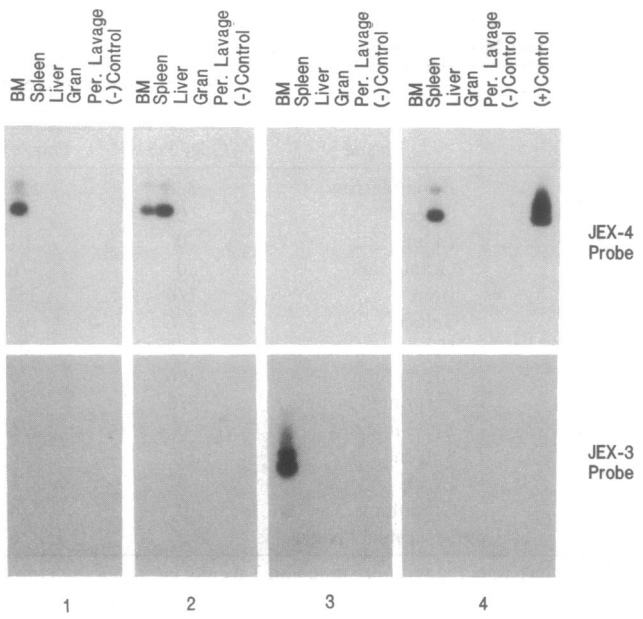

FIG. 5. gag–myb RNA in tissues of four mice 18 weeks after virus infection without pristane treatment. RT-PCR was as described. BM, bone marrow; Per. Lavage, peritoneal lavage. The same blot was hybridized with two different probes, as shown at right.

virus. Therefore, mice were inoculated with virus alone and compared with mice that received both virus and an earlier pristane inoculation. In the group receiving virus only, mice were seen to express gag–myb RNA as early as 2 weeks after virus infection. Overall there was a lower percentage of mice positive for the aberrant fusion message during the period from 3 to 8 weeks after virus infection (see Table 1). Yet, when four mice were examined at 18 weeks after virus infection, all were positive (Table 1 and Fig. 5) for RNA that hybridized with either JEX-3 or JEX-4 probes. In each case, the RNA was evident either in bone marrow or spleen tissue. In contrast to the group receiving pristane and virus, we never detected any mice in the virus-alone group that expressed RNAs positive for both probes.

Even though the above data suggest that pristane treatment increases the frequency of the insertional mutagenic event, other data indicate that for mice to develop disease, pristane is required to promote additional events. For example, mice that do not receive pristane do not develop disease (2) in spite of the fact that, as shown here, all mice are positive for c-*myb* insertional mutagenesis by 18 weeks even without pristane. In addition, most of the latency period of ≈100 days requires pristane. Table 3 shows that when mice are inoculated with virus and given pristane 2–16 weeks later, the latency period after pristane is about the same as the latency after virus if pristane is given before virus (Table 3).

Table 3. Time of pristane treatment relative to virus inoculation: Effect on tumor development

Pristane, week after virus	Incidence* (%)	Latency after virus inoc., days	Latency after pristane, days
−3	12/19 (63)	109	
+1	14/24 (58)	103	96
+2	10/19 (52)	114	100
+3	20/46 (43)	116	95
+5	8/20 (40)	142	107
+9	4/20 (20)	167	104
+16	5/20 (25)	207	95

All mice were inoculated i.v. with Mo-MuLV at 7–9 weeks of age; these mice were treated i.p. with 0.5 ml of pristane at the times indicated relative to virus infection. inoc., inoculation.
*Disease was assessed by examining smears of cells from the peritoneal cavity.

DISCUSSION

In the study reported here mice were examined during the preleukemic phase of MML for the evidence of cells that had undergone activation of c-*myb* by Mo-MuLV. With the use of RT–PCR such cells could be detected as early as 2–3 weeks after virus infection. Preneoplastic cells having undergone c-*myb* alteration were first evident in the bone marrow before their appearance in other hematopoietic organs. This result suggests, but does not prove, that the initial cells that have undergone insertional mutagenesis at this locus reside in the bone marrow. That the early leukemic or preleukemic cells are found predominantly at this site is consistent with the pathogenesis of human leukemia (17). The present data suggest also that during leukemogenesis cells are disseminated from the bone marrow to other hematopoietic organs, such as spleen and liver, as well as to the granulomatous tissue formed in the peritoneal cavity in response to pristane treatment. Preleukemic cells were not found at the latter site in nonpristane-treated mice.

We propose that the targeted cell, which is destined to became a leukemogenic promonocytic cell in MML and which undergoes c-*myb* activation by the virus, is a progenitor in the bone marrow. This cell is most likely a multipotential progenitor or granulocyte/macrophage progenitor. This hypothesis would be consistent with the fact that retroviruses preferentially integrate into genes that are actively being transcribed (18), and it is the progenitors that express c-*myb* (7, 8). The target cell is probably in the cell cycle at the time of proviral integration because stable retrovirus intermediates are probably not formed in stationary cells (19). The fact that cells must be replicating to become infected might help explain the increased frequency of integration at the c-*myb* locus seen at 3–8 weeks after virus infection in pristane-treated mice. We have already shown that pristane causes a stimulation of the myeloid compartment, even in hematopoietic organs outside the peritoneal cavity (4). The increased turnover of progenitors would provide a larger pool of cells in which c-*myb* could be activated by insertional mutagenesis. Our study does not address the state of differentiation of preleukemic cells in the various hematopoietic compartments. If target cells for insertional mutagenesis are truly progenitor cells, as we propose, and the overtly leukemogenic cells seen during the late acute stage of disease are differentiated, then interesting questions for further study are when do they differentiate and in what tissues.

Even though pristane may have some effect on increasing the frequency of c-*myb* activation, this effect could not be solely responsible for promoting leukemogenesis by Mo-MuLV in this system. Mice have never been shown to succumb to acute MML in the absence of pristane (2), in spite of the fact that 100% of mice receiving virus alone have cells that have undergone insertional mutagenesis by the virus (Table 1). Furthermore, as shown in Table 3, pristane is required during the entire disease latency period of ≈100 days. The pristane-induced inflammatory environment causes a tremendous influx of inflammatory cells, especially macrophages and neutrophils, into the granulomatous tissue throughout the peritoneal mesentery and peritoneal wall (20). Pristane may, therefore, promote the migration of preleukemic cells into the peritoneal cavity and/or provide growth-stimulating effects on cells once they have reached the granuloma. That insertion into c-*myb* is not sufficient for leukemogenic development is supported in the model of avian leukosis virus-induced induction of B-cell lymphomagenesis.

Pizer *et al.* (21) found that the incidence of c-*myb* activation or elevated expression of the protooncogene was much greater than the incidence of overt neoplastic disease.

Recently, we reported that splenectomy decreases MML incidence and the most dramatic effect is observed when splenectomy is done 4 weeks after virus infection. On the basis of that data and data presented here, we propose that a preleukemic cell in the bone marrow that has already undergone insertional mutagenesis at the c-*myb* locus most likely will pass through the spleen during an intermediate preleukemic phase before migrating to the peritoneal cavity.

In the group of mice that received virus but not pristane we have identified an early stage in the leukemogenic process that is blocked from progressing. In this early stage, sequestered cells that have a mutated oncogene exist, and these cells are waiting, poised to advance to the next transformation stage. The technique reported here for detecting preleukemic cells will now allow the study of mechanisms by which these preleukemic cells progress to a more transformed state and, perhaps, to a more differentiated state. Treatments can be evaluated in this system for their ability to mimic the effect of pristane and promote leukemogenesis.

Our technique for monitoring leukemic cells in the context of this acute myoblastic leukemia-like model also provides the opportunity for *in vivo* testing of drugs that suppress leukemia at various stages. Because the detection of cells is very sensitive, the model will allow one to monitor mice for low numbers of residual leukemic cells.

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