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Elevated Mutant Frequencies in Lymphoid Tissues Persist throughout Plasmacytoma Development in BALB/c.λLIZ Mice[1]

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ABSTRACT

Using the phage λLIZ-based transgenic *in vivo* mutagenesis assay, the mean mutant frequencies in the target gene, *lacI*, were found to be significantly increased in lymphoid tissues of congenic BALB/c.λLIZ N_5 mice in the terminal stage of a plasmacytoma induction experiment, 213–280 days after the first i.p. injection of the plasmacytomagenic agent pristane (2,6,10,14-tetramethylpentadecane). In plasmacytoma-bearing mice ($n = 7$), mutant frequencies in the spleens and mesenteric lymph nodes were elevated 2.46-fold and 5.35-fold, respectively, when compared with age-matched controls. In plasmacytoma-negative mice ($n = 11$), mutant frequencies were increased 2.30-fold (spleens) and 3.48-fold (mesenteric nodes). These results, interpreted in conjunction with our previous findings (K. Felix *et al.,* Cancer Res., *58:* 1616–1619, 1998) of approximately 3-fold elevations in pristane-induced splenic mutagenesis on day 42 postpristane, indicate that increased mutant levels in lymphoid tissues persist throughout plasmacytomagenesis in genetically susceptible BALB/c mice.

INTRODUCTION

Inflammation-induced peritoneal plasmacytomagenesis in mice provides a model system for studying many aspects of malignant B-lymphocyte development (1), including the role of chromosomal translocations that deregulate the proto-oncogene *c-myc* (2), the requirement for growth factors such as interleukin 6 (3), the effect of the environment (4), and the genetic determination of tumor resistance (5) and susceptibility (6). In the study presented here, we were interested in further elucidating the relationship between the genetic susceptibility to peritoneal plasmacytoma development and general mutagenesis in B cells. We hypothesized that in plasmacytoma-susceptible BALB/c mice the mutagenesis (mutant rates, mutant frequencies) in B cells obtained from mice undergoing tumorigenesis might be higher than the mutagenesis in B cells derived from normal, age-matched controls. Our hypothesis was based on the widely held belief that increased mutagenesis in the overall genome (*i.e.,* general mutagenesis) constitutes a critical and requisite companion of oncogenesis. However, the requirements for proving this proposition are not trivial; they include the availability of an experimental system for the accurate, quantitative evaluation of mutagenesis in the overall genome, as well as the means to assess mutagenesis in relevant biological samples, *i.e.,* in tissues with a substantial content of cells with a potential for transformation. We chose a transgenic *in vivo* mutagenesis assay (Big Blue, Stratagene) that is based on the phage λ-derived shuttle vector, λLIZ (7), to fulfill the first requirement, and the spleens and the mesenteric lymph nodes of mice primed to induce plasmacytomagenesis, to meet the second. Spleen and mesenteric node are major sites of B-cell proliferation and differentiation in the mouse. In addition, these tissues have been shown to contain putative plasmacytoma precursor cells that are defined as B cells harboring plasmacytoma-specific, *c-myc*-deregulating chromosomal translocations that can be detected by PCR analysis (8). Here, we show that general mutagenesis in the spleen and mesenteric node was significantly elevated in congenic BALB/c.λLIZ N_5 mice undergoing plasmacytoma development. Furthermore, we demonstrate that the increased *lacI* mutant rates in lymphoid tissues were maintained through the terminal stage of tumorigenesis, *i.e.,* for 3–6 months after the last i.p. injection of the plasmacytomagenic agent, pristane, had been given. These findings extend a previous study in BALB/c.λLIZ N_5 mice, in which an approximately 3-fold induction of splenic mutagenesis was demonstrated at a much earlier stage of tumorigenesis, on day 42 postpristane (9). Our combined results suggest that elevated mutagenesis in lymphoid tissues persists throughout plasmacytomagenesis in genetically susceptible BALB/c mice, providing support for the above-postulated link between general mutagenesis (in lymphoid tissues and B cells) and oncogenesis (lymphomagenesis) in the BALB/c plasmacytoma model.

MATERIALS AND METHODS

Derivation of BALB/c.λLIZ N_4 Mice. All of the mice were maintained in our conventional mouse colony at PerImmune, Inc. (Rockville, MD) under National Cancer Institute contract N01-BC-21075. The mice were generated by backcrossing a C57BL/6-derived region of Chr 4 carrying approximately 40 copies of the λLIZ transgene (10) onto inbred BALB/cπ SPF mice (hereafter called BALB/c mice). To facilitate the backcrossing, a protocol was used that combines the detection of the λLIZ transgene by PCR with the monitoring of the transmission of the paternal Chrs by means of SSLPs.[3] SSLPs were detected by PCR using commercially available primer pairs (Research Genetics, Huntsville, AL) chosen to screen for allelomorphic differences between BALB/c and C57BL/6 on each autosome and on the X Chr. This protocol permitted the identification of passenger segments from the C57BL/6 donor strain. Seventy-one markers were used to cover at least one centromeric, central, and telomeric portion of each Chr. At generation N_4, B/c.λLIZ mice were found to carry C57BL/6 alleles in regions of the genome that were not linked to the part of Chr 4 containing the phage λ transgene. Such alleles were detected in the centromeric region of Chr 7 (involving the anonymous marker Mit55) and the distal part of Chr 13 (involving the anonymous marker Mit78). Before SSLP analysis of transgene-positive offspring, a sample of genomic DNA was tested for packaging efficiency of the phage λ shuttle vector to assure that only animals in which the mutagenesis assay worked properly were considered for further breeding. The functional test was important, because it resulted in the identification (and exclusion from breeding) of mice in which the packaging efficiency was severely reduced. The reason for inefficient packaging is unclear but may be associated with mutations in the genomic region harboring the transgenic concatamer.

Expansion of BALB/c.λLIZ N_5 Mice for Plasmacytoma Induction Study. To test the susceptibility to pristane-induced plasmacytoma development, mice were expanded at the 5th backcross generation. Four "production cages" were set up in which a total of 10 BALB/c.λLIZ N_4 mice that were hemizygous for the λLIZ transgene ($λLIZ^{+/-}$) were bred with normal inbred BALB/c mice. A sample of tail DNA obtained from all 10 of the $λLIZ^{+/-}$ mice was tested for packaging efficiency of the phage λ shuttle vector as described

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[3] The abbreviations used are: SSLP, simple sequence-length polymorphic marker; Chr, chromosome; pfu, plaque forming unit(s).

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above. From the BALB/c.λLIZ N_4 × BALB/c crosses, 99 offspring were generated, of which 46 harbored λLIZ ("BALB/c.$\lambda LIZ^{+/-}$ N_5" mice) and 53 did not ("BALB/c.$\lambda LIZ^{-/-}$ N_5" mice). Thus, the transmission efficiency of the hemizygous Chr 4-derived transgenic segment from generation N_4 to generation N_5 was consistent with a Mendelian inheritance pattern (50% expected *versus* 46.5% observed) and not significantly reduced as previously reported by other investigators in a F1 cross using C57BL/6 mice hemizygous for λLIZ (11). Three transgenic mice were eliminated shortly after weaning, leaving 43 $\lambda LIZ^{+/-}$ mice to be included in the plasmacytoma induction study. Although more BALB/c-like than their N_4 ancestors, the generated BALB/c.λLIZ N_5 mice were not yet genetically pure and were likely to be characterized by residual heterozygosity originating from Chr 7- and Chr 13-derived C57BL/6 alleles. However, these mice were assigned to the tumor induction experiment because it is very unlikely that strain C57BL/6 carries major plasmacytoma resistance genes on Chrs 7 and 13 (12). The 4-week-old BALB/c.λLIZ N_5 mice were used for plasmacytoma-induction studies without further testing of phage λ packaging efficiency.

Induction and Diagnosis of Plasmacytomas. Malignant plasma cell tumors were induced in a total of 43 4-week-old BALB/c.$\lambda LIZ^{+/-}$ N_5 mice and 53 age-matched BALB/c.$\lambda LIZ^{-/-}$ N_5 mice with three i.p. injections of 0.2 ml pristane (2,6,10,14-tetramethylpentadecane) on days 1, 60, and 120. The control with syngeneic transgene-negative mice (BALB/c.$\lambda LIZ^{-/-}$ N_5) is important because of the substantial variations in plasmacytoma incidence values (ranging from 30 to 65%) that can be observed among various induction studies and presumably due to environmental factors that modify the penetrance of the genetic tumor-susceptibility phenotype. Beginning on day 150, Wright's Giemsa-stained cytospin slides of ascites containing peritoneal exudate cells were examined for the presence of large, hyperchromatic, atypical plasma cells, which can be used as reliable indicators of incipient plasmacytomas. Mice were scored as plasmacytoma-positive when 10 or more atypical plasma cells were detected per slide and when this observation was reproduced with an ascites sample obtained in the next round of cytospins. These reexaminations were scheduled on days 171, 192, 213, 234, 255, and 276 after the first application of pristane. The experiment was terminated at 280 days postpristane, at which day all of the remaining mice were killed. To avoid false negatives with respect to tumor development, the mesentery with attached granulomatous tissue was obtained from all of the mice scored as tumor-free on the basis of the previous cytological evaluations. The tissues were fixed, processed for histological analysis, and stained with H&E to allow the detection of incipient plasmacytomas that may not have been sufficiently expanded at day 280 to be discerned by cytological analysis. Thus, all of the plasmacytoma-negative mice listed in Table 1 were confirmed to be, indeed, devoid of tumors.

Determination of Mutant Frequencies. After killing the mice, spleens and mesenteric lymph nodes were flash-frozen in liquid nitrogen. High-molecular-weight genomic DNA was prepared using the Genomic DNA isolation kit from Qiagen, solubilized in TE buffer [10 mM Tris (pH 8.0) 0.1 mM EDTA] and stored in the dark at 4°C. The λLIZ transgenes were recovered as infectious phages by mixing 6 μg of mouse genomic DNA (*i.e.,* 12 μl of a preparation adjusted to 500 μg/ml) with Transpack packaging extract (Stratagene). Restriction-minus, *recA*, *lacZΔM15 Escherichia coli* SCS-8 host cells were then infected with recovered phage and plated out in top agarose containing 1.5 mg/ml 5-bromo-4-chloro-3-indolyl-β- D-galactopyranoside. After incubation for approximately 18 h at 37°C, the total number of plaques was

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Table 1 *Mutant frequencies of gene lacI in the spleen and mesenteric lymph node of plasmacytoma-positive and -negative mice*

All of the mice were obtained from the same group of congenic/transgenic BALB/cAnPt.$\lambda LIZ^{+/-}$ N_5 mice that were derived as described in "Materials and Methods." Mice 1–18 were treated with three i.p. injections of pristane to induce the development of plasmacytomas. Mice 19–22 were not treated and were used as age-matched controls for the pristane-treated animals. Mice 1–7 developed peritoneal plasmacytomas as a result of the treatment with pristane. Mice 8–18 did not develop tumors despite the same treatment. This result indicates the incomplete penetrance of plasmacytoma susceptibility, which is reflected by a tumor incidence of ≤65% in inbred BALB/c mice.

				Spleen					Mesenteric lymph node				
				Plaques, pfu					Plaques, pfu				
Mouse number	Laboratory code and gender	PCT[a]	Day[b]	Blue[c]	Total[d] $\times 10^5$	MF[e] $\times 10^{-5}$	MF[f] $\times 10^{-5}$	SD[g]	Blue	Total $\times 10^5$	MF $\times 10^{-5}$	MF $\times 10^{-5}$	SD
1	13-40 F	+	255	16	0.711	22.5			ND[h]	ND	ND		
2	30-94 M	+	276	5	1.05	4.78			ND	ND	ND		
3	08-25 F	+	234	7	1.06	6.61			ND	ND	ND		
4	25-77 F	+	276	8	1.11	7.18			ND	ND	ND		
5	23-72 M	+	255	19	1.25	15.2			174	0.401	433.0[i]		
6	06-19 F	+	213	11	0.975	11.3			18	0.992	18.1		
7	14-46 M	+	234	11	1.19	9.21			20	0.847	23.6		
							11.0	6.13				20.9	3.89
8	13-39 F	−	280	9	0.692	13.0			ND	ND	ND		
9	14-43 M	−	280	4	0.769	5.20			ND	ND	ND		
10	09-26 M	−	280	4	0.867	4.61			ND	ND	ND		
11	08-23 F	−	280	6	0.858	6.99			ND	ND	ND		
12	12-35 F	−	280	6	0.661	9.08			ND	ND	ND		
13	23-71 M	−	280	4	0.713	5.61			ND	ND	ND		
14	04-12 M	−	280	9	0.894	10.1			13	1.05	12.3		
15	11-33 M	−	280	7	0.826	8.48			15	0.873	17.2		
16	06-18 F	−	280	17	1.12	15.2			17	1.25	13.6		
17	03-09 M	−	280	13	0.702	18.5			8	1.00	7.97		
18	01-04 F	−	280	10	0.622	16.1			16	0.950	16.8		
							10.3	4.78				13.6	3.76
19	Co. 1 M	−	276	5	1.11	4.52			ND	ND	ND		
20	Co. 2 F	−	276	6	1.20	5.00			ND	ND	ND		
21	Co. 3 M	−	280	3	1.15	3.47			9	2.08	4.32		
22	Co. 4 F	−	280	6	1.22	4.92			3	0.858	3.49		
							4.48	0.704				3.91	0.587

[a] PCT, plasmacytoma; ND, not determined; Co., control.

[b] The number indicates the day after the first injection of pristane at which the mice were killed. Add approximately 30 days to determine the absolute age of the animals. The clock for the untreated age-matched controls (mice 19–22) was started together with the pristane-treated mice (1–18) for better comparison. Thus, mice 19–22 were 30 days older than indicated in the table.

[c] Number of blue plaques (pfu) with mutations in gene *lacI*.

[d] Number of total plaques, *i.e.*, blue mutant and colorless wild-type plaques, as multiples of 10^5.

[e] Mutant frequency determined as ratio of mutant:total plaques, as multiples of 10^{-5}.

[f] Mean mutant frequency in plasmacytoma-positive, plasmacytoma-negative, and control mice.

[g] SD of the mean mutant frequency.

[h] Not determined because the mesenteric node could not be retrieved (see the third paragraph of the "Results" section).

[i] The sample was excluded from calculating the mean mutant rate in mesenteric lymph nodes of plasmacytoma-bearing mice because the mutant level was unusually high and, therefore, was considered an outlier (see the fourth paragraph of the "Results" section).

determined and usually found to range from 6 to 40 × 10^3 plaques per packaging reaction. Blue plaques representing $LacI^-$ phages were carefully counted and then scored for mutant verification by replating. To avoid the loss of those *lacI* mutants that expressed the phenotype of very light blue plaques, the CM0 and CM1 faint color mutants (Stratagene) were used as internal sensitivity standards of the assay (13). Mutant frequencies were calculated as the ratio of $LacI^-$ phage/total ($LacI^+$ + $LacI^-$) phage and compared with the same ratio in the absence of such treatment, reflecting background mutant frequency.

RESULTS

BALB/c.λLIZ Mice Are Susceptible to Pristane-induced Plasmacytomagenesis. A total of 15 of 43 BALB/c.$\lambda LIZ^{+/-}$ N_5 mice developed pristane-induced peritoneal plasmacytomas by 276 days after tumor induction (the termination point of the experiment), a tumor incidence of 34.9%. The tumor rate observed in control, nontransgenic BALB/c.$\lambda LIZ^{-/-}$ N_5 mice was slightly higher; 22 of 53 mice developed plasmacytomas, a 41.5% incidence (Fig. 1). The difference between the strains of mice was not significant in the χ^2 test (P = 0.507), which led us to conclude that the transgenic $\lambda LIZ^{+/-}$ mice were as susceptible to plasmacytomagenesis as the control $\lambda LIZ^{-/-}$ mice. Nine BALB/c.$\lambda LIZ^{+/-}$ mice that developed plasmacytomas 213–276 days after the first injection of pristane were originally selected for the assessment of splenic mutant rates, the initial focus of this study (Table 1, columns 3 and 4). Unfortunately, the spleens from two of these plasmacytoma-bearing mice could not be included in the analysis because the mutagenicity assay did not function in these mice. In both of the animals, the ability to package the λLIZ shuttle vector was reduced by three orders of magnitude and, therefore, almost completely lost; only 3 and 13 pfu were obtained from an entire phage λ packaging reaction, whereas 6 × 10^3 to 4 × 10^4 pfu were recovered from the remaining seven mice using the same packaging reaction. The reason for the unexpected severe drop in packaging efficiency is currently unclear, but it is noteworthy that the same phenomenon was encountered before, during the derivation of the BALB/c.λLIZ N_4 mice (see the first paragraph in the "Materials and Methods" section). The spleens from 11 tumor-free BALB/c.$\lambda LIZ^{+/-}$ mice were obtained at day 280 postpristane to compare splenic mutagenesis among plasmacytoma-positive and -negative mice.

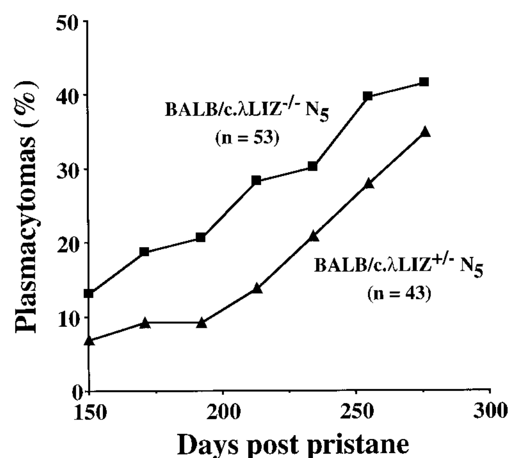

Fig. 1. Time course of plasmacytoma development in BALB/c.λLIZ N_5 mice. The total tumor incidences at day 276 postpristane were compared between transgenic $\lambda LIZ^{+/-}$ mice and nontransgenic $\lambda LIZ^{-/-}$ mice by 2 × 2 χ^2 analysis. No significant difference was found (P = 0.507). However, the inspection of the time course of tumor development suggested an apparent delay in tumorigenesis in the $\lambda LIZ^{+/-}$ group of mice, which was particularly manifest in the 150-to-213-day interval postpristane (*the first four data points in the tumor incidence curves*). This delay, which resulted in a significant difference when Wilcoxon's signed rank test was used to compare the incidence curves in $\lambda LIZ^{+/-}$ and $\lambda LIZ^{-/-}$ mice (P = 0.018), may have been caused by the presence of Chr 4-derived *C57BL/6* alleles in the vicinity of the transgenic integration site on Chr 4 in the $\lambda LIZ^{+/-}$ strain. Strain C57BL/6 is known to be resistant to pristane-induced plasma cell tumor development and may carry resistance genes in that region on Chr 4 (12).

Elevated Mutant Frequencies in the Spleen at the Terminal Stage of a Plasmacytoma-Induction Study. Splenic mutant rates were determined with the aid of the bacteriophage λ-based transgenic shuttle vector system (λLIZ) harboring *lacI* and *lacZ* as target and reporter genes of *in vivo* mutagenesis, respectively. In the experiment, two basic questions were asked: (*a*) whether the treatment with pristane resulted in persistently increased mutant levels in the spleen throughout the terminal stage of plasmacytomagenesis; and (*b*) whether the splenic mutant levels were higher in plasmacytoma-bearing mice than in mice in which no tumor developed. The result of the determination involving a total of 18 mice is summarized in Table 1 and Fig. 2. Fig. 2*B* shows that elevations in mean mutant rates over the background mutant levels of age-matched controls were observed in both plasmacytoma-positive mice (2.46-fold increase) and plasmacytoma-negative mice (2.30-fold increase). When both groups of pristane-treated mice were combined (n = 18) and compared with the untreated control group (n = 4), a highly significant (P = 0.001) 2.35-fold elevation of the mean mutant frequency was found. This finding suggested that BALB/c mice had an intrinsic proclivity to sustain enhanced mutant frequencies in the spleen for several months after the last injection of pristane had been given. In addition, the result extended our previous observations on the genotoxicity of pristane (14, 15), which is now firmly established as an *in vivo* mutagen in BALB/c mice. The increases in mean mutant levels in plasmacytoma-bearing and plasmacytoma-negative mice were not significantly different (P = 0.117). Thus, splenic mutagenesis was apparently not correlated with the appearance of plasmacytomas. The reason why splenic mutagenesis was a poor predictor of tumor appearance becomes clear when the variation of mutant levels in the spleen is inspected in Fig. 2*A*: some plasmacytoma-bearing mice showed relatively low mutant rates and some plasmacytoma-free mice displayed relatively high mutant frequencies.

Increased Mutant Levels in Mesenteric Lymph Nodes. To complement the determination of mutant rates in the spleen with the assessment of mutagenesis in another lymphoid tissue, attempts were made to obtain the mesenteric lymph nodes from the 18 mice from which the spleens were obtained. The mesenteric node was chosen because of its size and retroperitoneal location. These considerations were important for several reasons. The λLIZ-based mutagenicity assay requires approximately 20–30 μg of genomic DNA from hemizygous tissues, which can be obtained from the relatively large, elongated mesenteric node but not from small lymph nodes like peripheral (*e.g.,* inguinal or axillary) lymph nodes or Peyer's patches. The retroperitoneal location provides an anatomical barrier from the direct assault of the local inflammatory processes in the peritoneal cavity provoked by the i.p. injections of pristane. In only a fraction of mice was the dissection of the mesenteric node successful; it was obtained from 3 of 7 plasmacytoma-bearing mice and 5 of 11 plasmacytoma-negative mice. The incomplete recovery was caused by difficulties in finding and dissecting the node in mice 213 to 280 days postpristane. In these animals, the prolonged inflammation in the abdominal cavity frequently joined the mesentery, numerous inflammatory granulomata, gut loops, and other abdominal organs in a compact, sometimes calcified tissue mass in which the mesenteric node was found to be buried and hard to retrieve. Nevertheless, an interesting result was obtained in the few mesenteric nodes that were isolated: the mean mutant rates in this sample were 5.35-fold and 3.48-fold increased over the background mutant level in plasmacytoma-bearing and plasmacytoma-negative mice, respectively. The dif-

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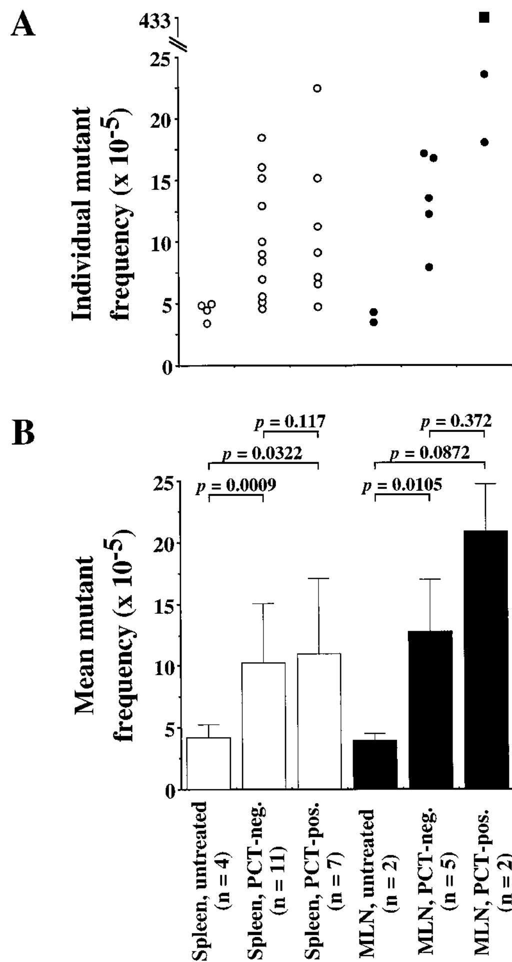

Fig. 2. Mutant frequencies in individual mice (*A*) and mean mutant frequencies in groups of mice (*B*) determined in the spleen and mesenteric lymph node (*MLN*) of congenic BALB/c.λLIZ$^{+/-}$ N_5 mice. Three groups of mice were compared: pristane-treated mice that developed a peritoneal plasmacytoma (*PCT-pos.*); pristane-treated mice that did not develop plasmacytoma (*PCT-neg.*); and age-matched controls that were not treated with the plasmacytoma-inducing agent pristane (*untreated*). *Brackets* on top of B labeled with the probability levels, the results of the statistical comparison of mean mutant rates performed with the two-tailed *t* test using the Statview software (Abacus Concepts, Berkeley, CA). One data point (■, *upper right hand corner of A*)—the mutant level observed in the mesenteric lymph node of a plasmacytoma-bearing mouse (mouse 5)—was excluded from statistical analysis because it reflected an outlier with the unusually high mutant frequency of 433×10^{-5}.

ference between the two groups was not significant ($P = 0.372$). However, when the tumor-bearing and tumor-free mice were combined ($n = 7$) and compared with their untreated, age-matched controls ($n = 2$), a significant ($P = 0.05$) 3.95-fold elevation in the average mutant rate of the mesenteric node was observed. This was similar to the increase seen in the spleen sample. Thus, the *lacI* mutant rates in the mesenteric node confirmed the observation in the spleen and suggested that treatment with pristane may result in the generalized mutagenesis throughout the lymphoid system.

Clonal Expansion of *lacI* Mutants in a Mesenteric Lymph Node. A startling observation was made in the mesenteric node of tumor-bearing mouse 5, in which the extremely high mutant level of 433×10^{-5} was encountered (Table 1, line 5; Fig. 2*A*, *data point to the upper right*). This value, which was considered an outlier and, therefore, excluded from the calculation of mean mutant frequencies in Table 1 and Fig. 2*B*, corresponded to the 111-fold elevation of the mean background mutant level determined in control lymph nodes (3.91×10^{-5}). Highly aberrant mutant levels like the one observed in mouse 5 are usually considered candidates for so-called jackpot mutations in gene *lacI* (16). Jackpot mutations are thought to be acquired during development and result in unusually elevated mutant rates in various tissues or cell lineages in the absence of genotoxic stress. However, this interpretation is unlikely for the sample described here because the mutant frequency of gene *lacI* in the spleen was found to be much lower (15.2×10^{-5}), albeit still 3.4-fold increased over the background (4.48×10^{-5}). Thus, the extraordinary enrichment of mutants seemed to be limited to the mesenteric node. The phenotype of the mutants was clearly heterogeneous as defined by the intensity of the blue color of mutant plaques; at least four distinct intensities ranging from light blue to dark blue were observed by visual inspection. This indicated the presence of distinct clonotypic mutations because the activity of β-galactosidase (determining blue color intensity) is known to be dependent on the type and location of the mutation in *lacI* (13). To explain the apparent presence of distinct, clonally expanded *lacI* mutants, we propose that one of the main biological functions of lymph nodes provided the mechanism; *i.e.,* the clonal expansion of B lymphocytes in the course of the humoral immune response. We speculate that in the mesenteric node of mouse 5, B cells that harbored preexisting mutations in *lacIs*, or B cells that acquired *de novo* mutations in the gene, were expanded during the development of follicles and the formation of germinal centers.

DISCUSSION

The basic finding described in this paper is the prolonged increase in mutant frequencies in spleens and mesenteric lymph nodes from plasmacytoma-susceptible BALB/c.λLIZ N_5 mice treated with pristane. Elevated mutagenesis was observed using the phage λ-based shuttle vector, λLIZ, a useful tool to quantitate mutagenesis *in vivo*. The increased mutant frequencies were found at the terminal stage of tumor induction, 213–276 days postpristane in plasmacytoma-bearing mice ($n = 7$) and 280 days postpristane in plasmacytoma-negative mice ($n = 11$). Thus, approximately 3–6 months had elapsed after the last (third) injection of pristane had been administered (on day 120) before the tissues were harvested and the enhanced mutant levels were determined. This result suggested that, in BALB/c mice, increased mutant frequencies can persist for long periods of time. In both plasmacytoma-bearing and -negative mice, mutagenesis in the spleen was found to be nearly 2.5-fold enhanced when compared with untreated, age-matched controls. Of note, a similar, approximately 3-fold increase in splenic mutant levels was observed in a previously conducted short-term experiment in which the spleens of BALB/c.λLIZ N_5 mice were obtained much earlier during oncogenesis, 42 days after the single injection of pristane (9). The nearly identical increases in splenic mutagenesis during the early stage (42 days postpristane) and the terminal stage of tumor development (213–280 days postpristane) led us to conclude that pristane-induced elevations in mutant levels may be sustained in lymphoid tissues throughout plasmacytomagenesis in BALB/c mice.

The reason for the apparent persistence of pristane-induced elevations in mutant levels in lymphoid tissues of BALB/c mice is not known, but a number of biological factors need to be examined to explain this phenotype. These factors can be divided conceptually into: (*a*) those that result in the enhanced generation of mutants; and (*b*) those that effect the diminished removal of mutants. Unfortunately, the transgenic mutagenicity assays presently available, including the λLIZ-based assay used here, are not helpful for distinguishing the biological factors acting on the supply side from those acting on the elimination side of mutagenesis; they simply report the number of mutants present in a particular tissue at a particular point in time. Thus, additional methods need to be recruited to decide whether the elevated mutant rates in gene *lacI* were caused by the increased

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production of mutants, their ineffective elimination, or a combination of both. The principal parameters that may increase the production of *lacI* mutants include the compromised protection of DNA (*e.g.,* by the diminished antioxidative defense), heightened DNA damage (*e.g.,* via elevated endogenous oxidative stress), and insufficient DNA repair. In BALB/c mice, several lines of evidence point to a fundamental deficiency in DNA repair that appears to affect global repair (17–20) and gene-specific repair (21). Very little is known about BALB/c-typical defects in the protection of DNA and the putative proclivity to elevated DNA damage. Virtually nothing has come to our attention about the efficiency with which mutants are eliminated in BALB/c mice. The biological factors that may compromise the removal of mutated cells may involve the failure of mutants to undergo apoptosis, the inappropriate growth support of aberrant cells that may permit their clonal expansion, the diminished effectiveness of immune system-mediated clearance mechanisms for damaged cells, and many others. More study is clearly required to dissect the mechanism(s) of persistent mutagenesis in lymphoid tissues of BALB/c mice and to explain the strong genetic component of this phenotype that was uncovered in the already-mentioned short-term study in which pristane-induced splenic mutagenesis on day 42 postpristane was observed in plasmacytoma-susceptible BALB/c mice but not in plasmacytoma-resistant DBA/2N mice (9).

The penetrance of peritoneal plasmacytoma development in genetically susceptible BALB/c mice is incomplete and known to be modified by antiinflammatory agents (22, 23) as well as by environmental factors that influence the immune system (4, 24). The incomplete tumor penetrance was also documented in the induction experiment performed here in BALB/c.λLIZ N_5 mice, in which approximately 35% of the mice developed plasmacytomas by day 280 postpristane, and 65% did not. This situation permitted us to analyze separately the *lacI* mutant levels in the plasmacytoma-bearing and plasmacytoma-negative mice. Although both groups of mice showed clear pristane-induced elevations in mean mutant frequencies in spleen and mesenteric lymph nodes, no differences between tumor-bearing and tumor-free mice were detected. The latter observation seemed to indicate that a correlation between mutagenesis in lymphoid tissues and the appearance of plasmacytomas did not exist. However, this interpretion may not be true and deserves great caution because of the following considerations:

(*a*) according to the conventions in our laboratory, the plasmacytoma-induction experiment was terminated arbitrarily on day 280 postpristane; *i.e.,* at a time point when it cannot be excluded that some of the plasmacytoma-negative mice would have developed tumors later on. It is, therefore, questionable whether the distribution of the animals into groups of tumor-bearing and tumor-free mice was fully adequate. All of the tumor models with an incomplete penetrance of tumor susceptibility are characterized by tumor incidences of less than 100% irrespective of the termination point of the study and, thus, are burdened with the shortcoming that additional tumors will always be scored when the observation period is further extended. Although this situation has not been caused by an error in study design, it is nevertheless a factor that complicates attempts to associate mutagenesis with oncogenesis;

(*b*) mutagenesis in *lacI*, which was determined with an assay (λLIZ) that detects primarily point mutations (base substitutions) and small insertions/deletions (frameshifts), may not be the most relevant parameter for plasmacytoma development because the role of these mutations in the pathogenesis of peritoneal plasma cell tumors is currently unclear. Another class of mutations, chromosomal translocations, is on the other hand known to be important for BALB/c plasmacytomagenesis (1). Chromosomal translocations are caused by illigitimate recombination, a form of nonhomologous genetic exchange that is also believed to be responsible for generating large deletions. On the basis of this parallel, it could be argued that an *in vivo* mutagenesis assay that is capable of detecting large deletions may be more pertinent for studies on plasmacytoma development than the λLIZ assay. Fortuitously, a plasmid-based (pUR288) *in vivo* mutagenesis assay that seems to be suitable for detecting large deletions caused by clastogens has recently been developed (25), partially validated (26), and made widely available (The Jackson Laboratory, Bar Harbor, ME). This assay should be used in future experiments to explore the possibility that large deletions in lymphoid tissues have more predictive value than point mutations for the development of BALB/c plasmacytomas; and

(*c*) the fact that whole lymphoid tissues were used as samples for mutational analysis may also have contributed to the failure to distinguish *lacI* mutant levels between mice that harbored plasmacytomas and mice that did not. Mutagenesis in lymphoid organs may simply be too insensitive a parameter to predict accurately the occurrence of plasmacytomas. The spleen and mesenteric lymph node are complex tissues that contain, besides many other cell types, a variable amount of B cells that usually does not exceed 40–50%. This multilineage complexity may obscure the presence of greater elevations of mutant rates in the B-cell compartment of plasmacytoma-bearing mice when compared with their tumor-free equals. It is, therefore, necessary to determine in follow-up experiments the *lacI* mutant frequencies in purified B-cell populations instead of lymphoid tissues. Furthermore, plasmacytoma precursor cells, a subpopulation within the B-cell pool, are arguably the most desirable targets for comparing mutagenesis with plasmacytoma development; yet, they likely represent only a minuscule population in lymphoid tissues. It is possible that these precursor cells are characterized by a particularly high mutant frequency and hence contribute disproportionally to the overall mutant levels in tissues. Plasmacytoma precursors are thought to harbor a constitutively active *c-myc* gene (2, 27), which may facilitate mutagenesis by keeping the cells in the active cell cycle (a general link between mito- and mutagenesis is well established) and by causing a mutator phenotype (28–30) that results in elevated genomic instability and mutagenesis (31). These considerations are important because they offer a rationale for examining the possibility that the occurrence of plasmacytomas, which are not correlated with overall mutagenesis in lymphoid tissues, may still be correlated with general mutagenesis in B-cells or certain subpopulations of B cells, such as tumor precursors.

In summary, the results reported in this paper provide evidence that in the experimental tumor-induction model of peritoneal BALB/c plasmacytomas, increased mutagenesis is not solely present in the early stages of tumorigenesis but persists throughout plasmacytoma development. It is conceivable that the continuous presence of high mutation frequencies is required for the stochastic accumulation of mutations until they achieve the combination necessary for complete neoplastic transformation of plasma cells. This study contributes another example of the usefulness of transgenic shuttle vector-based mutagenesis assays to evaluate the link between overall mutagenesis and tumor development (32–36), but it also points out the difficulties with which the student of carcinogenesis is confronted when he attempts to interpret the mechanism of elevated tissue mutagenesis and its role in a specific neoplastic process.

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