Moderate Hypermutability of a Transgenic lacZ Reporter Gene in Myc-Dependent Inflammation-Induced Plasma Cell Tumors in Mice

Klaus Felix, Axel Polack, Walter Pretsch, Sharon H. Jackson, Lionel Feigenbaum, Georg-Wilhelm Bornkamm, and Siegfried Janz

ABSTRACT

Mutator phenotypes, a common and largely unexplained attribute of human cancer, might be better understood in mouse tumors containing reporter genes for accurate mutation enumeration and analysis. Previous work on peritoneal plasmacytomomas (PCTs) in mice suggested that PCTs have a mutator phenotype caused by Myc-deregulating chromosomal translocations and/or phagocyte-induced mutagenesis due to chronic inflammation. To investigate this hypothesis, we generated PCTs that harbored the transgenic shuttle vector, pUR288, with a lacZ reporter gene for the assessment of mutations in vivo. PCTs exhibited a 5.5 times higher mutant frequency in lacZ (40.3 ± 5.1 x 10^-6) than in normal B cells (7.36 ± 0.77 x 10^-6), demonstrating that the tumors exhibit the phenotype of increased mutability. Studies on lacZ mutant frequency in serially transplanted PCTs and phagocyte-induced lacZ mutations in B cells in vitro indicated that mutant levels in tumors are not determined by exogenous damage inflicted by inflammatory cells. In vivo studies with a newly developed transgenic model of inducible Myc expression (Tet-off/MYC) showed that deregulated Myc sensitizes B cells to chemically induced mutations, but does not cause, on its own, mutations in lacZ. These findings suggested that the hypermutability of PCT is governed mainly by intrinsic features of tumor cells, not by deregulated Myc or chronic inflammation.

INTRODUCTION

The cause and significance of cancer-associated mutator phenotypes present a controversial, unresolved problem in cancer research. One school of thought postulates that mutator phenotypes are a common and requisite feature of neoplasia. Those who hold this view propose that tumor initiation and progression require a variety of specific genetic changes, necessitating a mutagenic pace that exceeds the spontaneous mutation rate (1, 2). The increased level of genomic instability (e.g., microsatellite instability, loss of heterozygosity) within a wide spectrum of tumors (3) and the high frequency of silent (unselected) mutations in genes, such as the one encoding p53, have supported this view (4). An alternative school of thought believes that oncogenesis is an essentially evolutionary process driven by cellular selection, considered to be capable of causing cancer in the presence of normal (spontaneous, background) mutation rates (5). The holders of this view argue that the mutator phenotype hypothesis is largely a product of overgeneralization from rare genetic syndromes linked to raised mutation rates and cancer predisposition; e.g., hereditary non-polyposis colon cancer and ataxia telangiectasia. Furthermore, the growth of colorectal tumors and, presumably, many other common neoplasms begins with a normal mutation rate (6).

Mouse models of human cancer are suitable for studying the relationship between general mutagenesis and oncogenesis under genetically controlled conditions. Among the many advantages of the mouse is the ability to assess mutations in vivo using transgenic shuttle vectors with a bacterial reporter gene. Several transgenic reporter systems for measuring mutation frequency in vivo have been developed (7–11). Two widely used systems rely on the phage λ vector, ALIZ (12, 13), or the plasmid vector, pUR288 (14). Both vectors contain nontranscribed, neutral reporter genes (lacI in case of ALIZ and lacZ in case of pUR288) that have been incorporated into the mouse genome in which mutations occur. The reporter genes are excised from mouse genomic DNA and transferred to Escherichia coli, affording efficient selection, enumeration, and analysis of mutations. Both mutagenesis assays, ALIZ/lacI and pUR288/lacZ, are validated and thought to accurately reflect the mutant frequency (MF) in the overall genome (15, 16). One notable difference, however, is that the ALIZ/lacI assay detects primarily point mutations and small deletions in lacI, whereas the pUR288/lacZ assay additionally detects large deletions in lacZ and intra- and interchromosomal rearrangements of lacZ with mouse genomic sequences.

Elevated mutagenesis may facilitate the development of plasmacytomomas (PCTs), malignant tumors of terminally differentiated B lymphocytes. Peritoneal plasmacytomogenesis in mice is dependent on a number of pathogenetic factors (reviewed in Ref. 17), of which at least two have the potential to increase general mutagenesis in B cells and plasma cells. The first one is the chronic inflammatory, granulomatous tissue that is formed in response to i.p. injections with pristane, which are required to induce PCT in genetically susceptible BALB/c (C) mice. The mutagenic principle in the pristane granuloma is the resident population of inflammatory cells, mainly macrophages and neutrophils, which are able to mutate bystander B/plasma cells; e.g., by releasing oxygen intermediates into the extracellular milieu (18–20). The second factor is the chromosomal translocations that result in the deregulated expression of the oncogene c-Myc. Virtually all pristane-induced PCT carry Myc-activating translocations, most commonly T(12;15). Deregulated expression of Myc in consequence of T(12;15) translocation may cause increased mutagenesis by a variety of mechanisms, including (a) uncoupling of DNA replication from cell cycle progression (21, 22), (b) down-regulation of genes involved in DNA repair (e.g., members of the GADD family; Ref. 23) or antimutagenesis (e.g., BCL2; Ref. 24), (c) interference with DNA mismatch repair (25), and (d) disruption of redox homeostasis (26–28). The involvement of pro-mutagenic factors in PCT development, such as chronic inflammation and deregulated Myc, provided the rationale for the hypothesis that PCTs exhibit a phenotype of increased mutability (mutator phenotype).

Here we used the pUR288/lacZ mutagenesis assay to investigate whether PCTs have a mutator phenotype, and, if so, whether this phenotype may be caused by chronic inflammation and/or deregulated Myc. We thus backcrossed the pUR288 transgene from PCT-resistant C57BL/6 (B6) mice onto PCT-susceptible C mice and induced PCTs in the newly generated C.pUR288 congenics. We found that primary and transplanted PCTs exhibited the mutator phenotype of 5.5-fold
elevated MFs in lacZ compared with the background levels in normal B cells. To elucidate potential mechanisms of hypermutability, we designed in vitro model systems for assessing the mutagenic impact of inflammatory cells and activated Myc on primary B cells. The latter involved a new transgenic mouse model of inducible Myc expression, Tet-off/Myc. Although the mutation-enhancing effect of inflammatory cells and deregulated Myc was readily demonstrated, the aggregate of our findings suggested that the mutator phenotype of PCTs is mainly determined by intrinsic features of the tumor cells, presumably including mutator genes whose nature remains to be identified.

MATERIALS AND METHODS

Generation of C. pUR288 Mice. Strain C.pUR288 was generated by transferring the pUR288 transgene residing on chromosome (Chr) 3 (14) from C57BL/6 (B6) to BALB/cAnPt (C) using speed backcrossing. The original B6 mice carrying pUR288 (line 60) were developed by Dr. Jan Vijg, University of Texas, San Antonio. These mice harbor two virtually identical copies of the pUR288 transgene on Chrs 3 and 4. Both copies consist of approximately 10 individual pUR288 genes. The chromosomal integration site of the transgene residing on Chr 4 happens to be in a region that contains allelomorphic variants of tumor modifier genes that confer susceptibility to PCTs in strain C but resistance to PCTs in strain B6 (29). To avoid the risk of replacing the susceptibility alleles of C with resistance alleles from B6, the transgene on Chr 4 was not transferred onto C (Supplementary Fig. 1).6 The intentional loss of one copy of the transgene resulted in the deterioration of the performance of the pUR288/lacZ assay by approximately 50%. Thus, only half the number of pUR288 plasmids were excised from genomic DNA of C.pUR288 mice relative to parental B6.pUR288 mice.

The speed backcross protocol for the pUR288 transgene on Chr 3 combined the genotyping for pUR288 (PCR assay) with the monitoring of the transmission of paternal Chrs by simple-sequence-length polymorphic markers. The latter were identified by PCR using commercially available primer pairs (Research Genetics, Huntsville, AL). Eighty allelomorphic differences of strains C and B6 covering the centromeric, central, and telomeric portions of all autosomes and Chr X were screened to select the most appropriate breeders for the next generation backcross. At backcross generation N2, C.pUR288 were found to carry B6 alleles only in the 5′ and 3′ flanks of the pUR288 transgene on Chr 3. All mice were maintained in our conventional mouse colony at the National Cancer Institute under Animal Study Protocol LG-020.

Induction and Transplantation of PCTs. PCT were induced in 10-week-old C.pUR288 mice by three i.p. injections of 0.5 ml pristane spaced 2 months apart (30). Age-matched inbred C mice were used as controls. Beginning 1 week after the third injection of pristane, the mice were monitored for tumor development by microscopic examination of cytocyte specimens of ascites cells stained according to Wright’s Giemsa. Ascites samples (50–100 μl) were obtained by inserting a 16-gauge hypodermic needle into the peritoneal cavity. The presence of 10 or more, large, hyperchromatic, atypical plasma cells was indicative of incipient PCTs. The mice were scored as tumor-positive when the number of colonies. GaIE revertants were excluded from determinations of MF. To analyze lacI2 mutations, plasmid DNA was extracted from overnight cultures using QiAprep 96 Turbo Miniprep Kit (Qiagen, Valencia, CA). Point mutations were distinguished from rearrangements (R) by restriction analysis with HindIII, AvaI/PstI, and SacI/PstI (New England Biolabs). Point mutations demonstrated the wild-type pattern of pUR288, whereas R demonstrated a deviant pattern.

Mutagenesis in B Cells Exposed to PECs. LPS blasts were prepared from the spleens of 3-month-old C.pUR288 mice as described previously (20). Briefly, splenic perfusates were depleted of erythrocytes in hypotonic lysis buffer and were seeded at a density of 106 cells/ml in tissue culture flasks. The cells were grown for 48 h in RPMI 1640 supplemented with 50 μg/ml LPS from E. coli 055:B5 (Sigma, St. Louis, MO), 10% FCS, 2.0 mm l-glutamine, 50 μM 2-mercaptoethanol, and penicillin and streptomycin (Life Technologies, Inc.). Peritoneal exudate cells (PECs) were obtained from 3–6-month-old C mice primed with 0.5 ml of pristane 2–3 weeks before harvesting the PECs by peritoneal lavage. In some experiments, PECs were obtained from mice homozygous for a null allele of phox and the hypomorphic Gpdx allele of glucose-6-phosphate dehydrogenase (G6PD; Ref. 32). Loss of p47phox abrogates NADPH oxidase function, whereas partial deficiency of G6PD interferes with the oxidative burst by limiting the NADPH supply (Fig. 2E). The average yield of PECs was 1.5 × 107 per mouse. PECs consisted mainly of neutrophils (~60%) and macrophages (~40%) and were at least 95% viable as determined by trypan blue exclusion. To assess PEC-mediated mutagenesis in B cells, the pUR288-harboring LPS blasts were cocultured with PECs (not containing pUR288) at a ratio of 1:2 for 24 h at 37°C. 12-O-tetradecanoylphorbol-13-aceta- ta (TPA) (50 nm; LC Laboratories, Woburn, MA) was added to the cell culture (four applications spaced 6 h apart) to trigger the oxidative burst in PECs. After 24 h of coincubation at 37°C, the cells were harvested and genomic DNA was made for the pUR288 assay. In one experiment, LPS blasts were exposed to 4-nitroquinoline-1-oxide (4-NQO) in the absence of PECs (Fig. 2C).

Mutagenesis in B Cells with Inducible Myc Expression. To develop a system of inducible Myc expression in primary B cells, B6 mice harboring the Tet-off/MYC transgene were developed (Fig. 3A). The transgene consisted of an Miu-Pval fragment obtained from construct, pmyc-tet (BC266), described in greater detail elsewhere (33). The Tet-off/MYC contains the tetracycline-controlled transactivator, tTA, a minimal MYC promoter, and the protein-encoding portion of the human MYC gene (exons 2 and 3). The expression of MYC is activated on withdrawal of the tetracycline analog, doxycycline (Tet-off). Tet-off/MYC mice were crossed with B6.pUR288 mice to develop a system in which mutagenesis in primary B cells can be studied under conditions of inducible Myc expression. 12B20 splenocytes from Tet-off/MYC or pUR288 blasticogenic were fractionated by MACS (Miltenyi Biotech) using 2B20 (CD45R) VS + columns. Cell recovery relative to total splenocytes was ~90%. The purity of MACS-sorted B cells was ~90% by fluorescence-activated cell sorting. The pUR288-harboring B cells were cocultured in the absence of doxycycline (MYC off) or absence of doxycycline (MYC on). Myc expression was monitored by Western blotting. In some experiments, 4-NQO was added to induce heightened levels of mutagenesis compared with the pro-mutagenic environment of standard cell culture. Cells were harvested after 3 and 27 h to prepare genomic DNA for the pUR288 assay.

RESULTS

C.pUR288 Mice Are Susceptible to PCTs and Are Suitable for Evaluations of MFs. The genetic susceptibility to pristane-induced PCTs is a complex trait. Strain B6 and many other common inbred strains of mice are resistant to PCTs (34). In contrast, strain C is susceptible, which is caused, in part, by three susceptibility alleles on Chr 4 (35, 36). The transfer of genetic material from a PCT-resistant strain, such as B6, onto C involves the risk of contaminating the C genome with PCT resistance alleles, which may result in the decrease or even abrogation of PCT susceptibility. To evaluate whether the backcross of the pUR288 transgene from B6 onto C altered the susceptibility of C to PCTs, a tumor induction study with the newly generated C.pUR288 congenics was performed. Eight (40%) of 20 C.pUR288 mice developed PCTs by 260 days after the first injection.

6 Supplementary data: http://cancers.aacrjournals.org.
of pristane (Fig. 1A, squares). Pristane-treated C mice showed a similar incidence [7 (35%) of 20] and onset of PCTs (Fig. 1A, circles). These findings indicated that strain C.pUR288 is as susceptible to PCTs as strain C.

Compromising the performance of the pUR288/lacZ mutagenesis assay is another risk when generating pUR288 congenics. The lacZ assay is dependent on the integrity of the pUR288 transgene, which can be altered by deletions and other genetic rearrangements that result in the loss of individual gene copies from the concatamer. Loss of copy number affects assay performance by reducing the efficiency with which reporter genes can be excised from mouse genomic DNA and transferred to E. coli. This problem was encountered in a previous study involving the B6 to C transfer of the ALIZ transgene, in which a severe drop in phage packaging efficiency was noted at backcross generations N4 and N5 (37). To avoid similar problems during the B6 to C transfer of the pUR288 transgene, the performance of the pUR288/lacZ assay was monitored at each backcross generation (N1–N5) to select breeders with a properly functioning mutagenesis assay. The average plasmid rescue efficiency at generation N7 (used for the tumor induction study described above) was \( \sim 9 \times 10^7/\mu g \) DNA, \( \sim 50\% \) lower than in parental B6 mice. The drop in rescue efficiency was expected because, unlike B6 mice, C mice contained only one copy of pUR288. Despite the reduction in assay performance, strain C.pUR288 was suitable for determining MF in PCTs using the lacZ reporter gene assay.

**Elevated MFs in Primary PCTs.** The determination of the MF in transgenic reporter genes in tumors and corresponding normal and premalignant tissues provides important information about genomic stability control during oncogenesis. To evaluate whether pristane-induced PCTs exhibit genomic instability, the MF in lacZ was compared with that in B cells and peritoneal granulomas. This comparison was meaningful because B-lymphocytes are the precursors of PCTs and the inflammatory granuloma is the tissue site in which PCTs develop (Fig. 1B). The mean MF in primary PCTs (40.3 \( \times 10^{-5} \); \( n = 5 \)) was 5.5 times higher than the mean MF in MACS-sorted B220\(^+\) splenocytes (B cells; 7.36 \( \times 10^{-5} \)). This indicated that PCTs have a phenotype of moderate hypermutability. The MF in B220\(^+\) splenocytes was 2.2 times higher than in B220\(^-\) splenocytes (non-B cells), consistent with a previous result using ALIZ (38). The relatively high MF of splenic B cells may be related to their high proliferative activity in vivo, considering that increased proliferation facilitates mutagenesis (39). The MF in the granulomas (17.3 \( \times 10^{-5} \)) was 2.3 times lower than the MF in the PCTs, but 3.7-fold higher compared with the mesentery (4.71 \( \times 10^{-5} \)). The mesentery, a fatty tissue, was included as a control for the granulomas because it serves as the matrix for granuloma development in pristane-treated mice. The increased MF in the granulomas relative to the mesentery suggested that resident granuloma cells (mainly phagocytes, such as macrophages and neutrophils) accumulate somatic mutations by self-inflicted mutagenesis, possibly by releasing reactive oxygen intermediates. It further suggested that the granulomas provide a mutagenic environment for infiltrating B cells, which may become targets of phagocyte-mediated oxidative damage (Fig. 1B, top). However, the contribution of B cells to the elevated MF in the granulomas remained unclear, because mutations in B cells were not distinguished from mutations in granuloma cells (both cell types carried the lacZ reporter gene).

**Mimicking the Mutagenic Effect of the Inflammatory Granuloma in Vitro.** To measure mutations in B cells exposed to inflammatory granuloma cells, we cocultured LPS-stimulated B220\(^+\) splenocytes from C.pUR288 mice in vitro with pristane-elicited PECs (macrophages and neutrophils) from normal C mice. Mutations in the B cells (positive for lacZ) were enumerated, whereas mutations in PECs (negative for lacZ) were not (Fig. 2A). The coincubation of B cells and PECs for 24 h at a ratio of 1:2 resulted in a MF of 20.1 \( \times 10^{-5} \) in the B cells, a 3.2-fold increase compared with B cells without PECs (6.2 \( \times 10^{-5} \)). Activation of the oxidative burst in PECs (four applications of 50 nM TPA spaced 6 h apart) resulted in a small additional increase in the MF to 23.2 \( \times 10^{-5} \) but was not significant. Treatment of B cells with TPA in the absence of PECs caused an \( \sim 1.5\)-fold increase in MF (10.3 \( \times 10^{-5} \)) relative to background levels in untreated B cells (Fig. 2B). These results were consistent with previous ones using the ALIZ assay (20). The magnitude of PEC-induced mutagenesis was impressive compared with that induced by the powerful UV-mimetic mutagen, 4-NQO, which generates oxidative stress and DNA adduct formation through intracellular redox cycling (40). TPA-stimulated PECs and 25 nM 4-NQO were roughly equipotent (Fig. 2C). The addition of the antioxidant catalase
Fig. 2. Phagocytes mutate neighboring B cells in vitro. A, cytomorphology of cocultured LPS blasts and peritoneal exudate cells (PECs). The blasts harbored the shuttle vector pUR288, but the PECs did not. PECs consisted mainly of neutrophils (arrow pointing right) and macrophages (arrow pointing down). LPS blast included large activated cells (arrow pointing up) frequently exhibiting mitotic figures (arrow pointing left; H&E ×40). B, mean lacZ mutant frequency (MF) in LPS blasts after coinubcation with PECs either stimulated with TPA (column 4) or left nonstimulated (column 3). TPA-treated blasts (column 2) and untreated blasts (column 1) were included as controls. Vertical lines above the columns, SD of mean MF. The Ps were calculated in two-sided t tests. See legend to Fig. 1C for definition of Mutants, Total lacZ, Exp., MF, and SD. C, 4-nitroquinoline-1-oxide (4-NQO)-induced mutations in lacZ in LPS blasts. Cells were treated for 24 h in the presence of the indicated concentrations of 4-NQO. Asterisks above the columns, significant increases (p < 0.05) in MF relative to the background value in the untreated control (χ² test). MF calculation was based on a total of 784 mutants (range, 40–299 mutants) scored among 2.1 × 10⁶ wild-type colonies (range, 2.64 × 10⁵ to 5.59 × 10⁵). D, inhibition of PEC-mediated mutagenesis in B cells by the antioxidants, catalase [C (column 3)] or catalase plus pyruvate [C + P (column 4)]. Above the columns, residual lacZ MF (%) relative to TPA-stimulated PECs without antioxidants (100%, column 1). MF calculation was based on a total of 179 mutants (range, 20–76) scored among 8.07 × 10⁵ wild-type colonies (range, 122 × 10⁵ to 272 × 10⁵). E, genetic defects in oxidative burst leading to diminished mutagenic potential of PECs. PECs deficient in p47 phox, an essential component of active NADPH oxidase, are unable to produce superoxide (O₂⁻) in the NADPH oxidase reaction. O₂⁻ is further reduced to the highly reactive hydroxyl radical (not shown) and reacts with nitric oxide (NO) to form peroxynitrite (ONOO⁻), which is highly toxic compared with O₂⁻ and NO (68). Peroxynitrite is also a potent genotoxin (69). PECs with a partial deficiency in glucose-6-phosphate dehydrogenase (G6PD) due to the hypomorphic Gpdx⁻/m2Neu allele produce insufficient amounts of NADPH, which is used, among other enzymes, by NADPH oxidase and the inducible nitric oxide synthase (iNOS). Thus, although G6PD-deficient PECs have a partial but compound defect in generating mutagenic oxygen and nitrogen species, p47 phox null PECs have a complete defect in one pathway of reactive oxygen intermediate production. F, partial loss of mutagenic potency in p47 phox null PECs (P) and G6PD-deficient PECs (G) relative to normal PECs (C) obtained from C mice. The experiment enumerated 682 mutants (range, 35–224) in a total of 6.22 × 10⁵ colonies (range, 3.84 × 10⁵ to 2.21 × 10⁵).

To overexpress a human Myc gene in all tissues, including B cells, on withdrawal of the tetracycline analogue, doxycycline (Fig. 3A). Fig. 3B (top) shows the time course of Myc protein expression in Tet-off/MYC-pUR288 bitransgenic B cells on withdrawal of doxycycline. The increase in Myc reached a maximum after ~3 h and lasted for 27 h. A similar peak of Myc expression at 2–4 h was also observed in SV40 large T-transformed Tet-off/MYC-pUR288 fibroblasts used as controls (Fig. 3B, bottom).

To evaluate mutagenesis in B cells under conditions of inducible Myc expression, MACS-sorted LPS-stimulated bitransgenic B220⁺ splenocytes were cultured in the presence or absence of doxycycline before preparing genomic DNA for the lacZ mutagenesis assay. Some cell cultures were exposed to oxidative cell culture stress and others were treated with 4-NQO to generate additional genotoxic stress (Fig. 3C). Treatment with 4-NQO resulted in a significantly higher MF in Myc-overexpressing B cells (MYC on) compared with normal B cells (MYC off) at 27 h (88.6 × 10⁻⁵ versus 41.8 × 10⁻⁵), indicating that deregulated Myc can sensitize B cells to undergo elevated mutagenesis in the presence of a strong mutagen. The difference was much smaller at 3 h (20.8 × 10⁻⁵ versus 18.6 × 10⁻⁵), suggesting that the mutation-enhancing effect of overexpressed Myc was dependent on time (cell division) to fix the mutations. Under conditions of relatively...
MUTATOR PHENOTYPE IN MOUSE PCTs

Fig. 3. Myc sensitizes B cells to chemically induced mutagenesis. A, scheme of the Tet-off/MYC transgene. The transactivator, tTA, is driven by the constitutively active, minimal cytomegalovirus (CMV) promoter and, thus, is always produced. In presence of doxycycline (small gray circles binding to tTA), tTA cannot dock onto the tetO7-TP element, which includes the Myc promoter. On withdrawal of doxycycline, tTA binds to tetO7-TP, which results in the expression of Myc mRNA and protein (33, 70). B, time course of Myc protein expression on withdrawal of doxycycline in LPS blasts (top) and a fibroblast line (bottom) transformed by the SV40 large-T antigen. Shown are Western blots of lysates of B-lymphoblasts developed with the horseradish peroxidase-conjugated Myc antibody 9E10 (1:100) or lysates of fibroblasts developed with the polyclonal antibody A-14 (1: 500). Both antibodies were from Santa Cruz Biotechnology. Actin, which was used as loading control, was detected with an antibody from Sigma. C and D, lacZ mutant frequency (MF) in B cells and fibroblasts overexpressing Myc (on) or not overexpressing Myc (off). Cells were treated with 50 nm 4-nitroquinoline-1-oxide (4-NQO) for the indicated time (+) or left untreated (−). The MF in the fibroblasts are given in relative units, using the background MF in untreated cells as the reference point (100%). The background MF was 1.7 × 10−5, which was unusually high. The reason for this is not known but is possibly related to the origin of the cell line, which was derived from primary C.pUR288 fibroblasts by transformation in vitro with large T antigen from SV40 virus (using plasmid, pSV40-T Ag), followed by transfection with the Tet-off/MYC expression vector (LipofectAMINE, Boehringer Mannheim) and selection in 100 μg/ml hygromycin. The background MF of other cell lines obtained by this protocol was even higher (results not shown). Large T antigen is a potent mutator gene in mice (71). See legend to Fig. 1C for definition of Mutants, Total lacZ, and MF.

Mutant Levels and Patterns of Mutations in Transplanted PCTs. Transgenic reporter genes are useful to evaluate whether in vivo propagation of primary tumors leads to run-away genomic instability (13, 46–49). To investigate this possibility, we serially transplanted (G1–G3) four primary PCTs (G0) into pristane-primed C mice to undergo R mutations is the weak allele of DNA-PK has recently been shown to be a deviant pattern. Increases in R mutations, which include large deletions that extend into the mouse genome, deserve particular attention, because R mutations may be underrepresented in the pUR288/lacZ assay. R mutations are probably at a selective disadvantage whereas point mutations are not (51). In lacZ mutants obtained from PCTs, R mutations were clearly the dominant category of mutations [132 (72.5%) of 182; Fig. 4B]. R mutations were less prevalent in B cells exposed to PECs [44 (63.7%) of 69], in B cells from the Tet-off/MYC experiment [490 (54.5%) of 868], and in control tissues [145 (51%) of 284]. The prevalence of R mutations in normal tissues of C.pUR288 mice (51%) was higher than that reported for B6.pUR288 mice (42–45%; Refs. 14, 52), which may reflect genotypic differences in genomic stability control between strains C and B6. One of the genes that may contribute to the apparent proclivity of C mice to undergo R mutations is the weak allele of Prkdc encoding the catalytic subunit of the DNA double-strand break repair protein, DNA-PK, a key player in DNA double-strand break repair (53). The weak efficiency allele of DNA-PK has recently been shown to be associated with delayed genomic instability in B cells (54) and increased lymphoma susceptibility (55).

DISCUSSION

The present study describes a mutator phenotype in a mouse model of neoplastic plasma cell development that is tightly associated with chronic inflammation and activated Myc. The mutator phenotype of

mild oxidative damage associated with cell culture stress, the MF in Myc-overexpressing B cells was lower relative to normal B cells: 11.9 × 10−5 versus 14.7 × 10−5 at 3 h and 17.2 × 10−5 versus 20.0 × 10−5 at 27 h. This result demonstrated that overexpressed Myc, on its own, is not a mutator gene in B cells. Myc-dependent changes in lacZ mutant levels in fibroblasts were strikingly similar to those observed in B cells (Fig. 3D). In the absence of 4-NQO, the MF was higher (2.4-fold) in Myc-overexpressing fibroblasts relative to normal fibroblasts (Fig. 3D, compare columns 4 and 2). In the absence of 4-NQO, the MF in Myc-overexpressing fibroblasts was lower (by 18%) than in normal fibroblasts (compare columns 3 and 1). The matching results in two different cell types (B cells and fibroblasts) indicated that deregulated Myc might act as a catalyst of chemically induced mutagenesis in many tissues. Furthermore, the mutation-enhancing ability of Myc might be fully compensated when genotoxic stress is low.

Mutant Levels and Patterns of Mutations in Transplanted PCTs. Transgenic reporter genes are useful to evaluate whether in vivo propagation of primary tumors leads to run-away genomic instability (13, 46–49). To investigate this possibility, we serially transplanted (G1–G3) four primary PCTs (G0) into pristane-primed C mice and harvested cells 4–6 weeks posttransplantation. Pristane treatment of recipient mice was required for the outgrowth of transfected tumor cells, which are as dependent on the microenvironment of the pristane granuloma as are primary PCTs (50). The MF in G1 to G3 PCTs was remarkably stable (42.3–47.0 × 10−5; Fig. 4A) and only slightly higher than the MF in primary PCTs (40.3 × 10−5). This demonstrated that mutant levels in PCTs were not determined by the length of stay in the granuloma (35 ± 7 days for transplanted PCTs versus 180 ± 45 days for primary PCTs). Furthermore, unlike primary PCTs in which mutations in granuloma cells may have contributed to the overall MF in the tumors, only mutations in tumor cells were measured in the transplanted PCTs. This added confidence to the contention that serial propagation of PCTs was not characterized by elevated genomic instability and that PCT mutant levels were governed mainly by intrinsic properties of the tumor cells.

Analysis of mutational patterns in transgenic reporter genes can provide important mechanistic insights into tumor-associated mutator phenotypes. The pUR288/lacZ assay affords the opportunity to distinguish two important classes of mutations by simple restriction analysis of lacZ mutants. Point mutations are identified by the wild-type pattern of pUR288, whereas rearrangements (R) are identified by a deviant pattern. Increases in R mutations, which include large deletions that extend into the mouse genome, deserve particular attention, because R mutations may be underrepresented in the pUR288/lacZ assay. R mutations are probably at a selective disadvantage whereas point mutations are not (51). In lacZ mutants obtained from PCTs, R mutations were clearly the dominant category of mutations [132 (72.5%) of 182; Fig. 4B]. R mutations were less prevalent in B cells exposed to PECs [44 (63.7%) of 69], in B cells from the Tet-off/MYC experiment [490 (54.5%) of 868], and in control tissues [145 (51%) of 284]. The prevalence of R mutations in normal tissues of C.pUR288 mice (51%) was higher than that reported for B6.pUR288 mice (42–45%; Refs. 14, 52), which may reflect genotypic differences in genomic stability control between strains C and B6. One of the genes that may contribute to the apparent proclivity of C mice to undergo R mutations is the weak allele of Prkdc encoding the catalytic subunit of the DNA double-strand break repair protein, DNA-PK, a key player in DNA double-strand break repair (53). The weak efficiency allele of DNA-PK has recently been shown to be associated with delayed genomic instability in B cells (54) and increased lymphoma susceptibility (55).
peritoneal PCT was characterized by a 5.5-fold increase in the MF of a transgenic lacZ reporter gene relative to the MF in normal B cells, and a preponderance of rearrangement mutations of lacZ (~75%) in the tumor cells. To better understand this mutator phenotype, we evaluated the mutagenic consequences of inflammation and the deregulated Myc expression in B cells with the help of two dedicated in vitro model systems. The first model used the coinoculation of B cells and PECs to mimic the mutagenic environment of the inflammatory granuloma. It showed that macrophages and neutrophils are able to mutate neighboring B cells, in part by releasing reactive oxygen intermediates. The second model used the Tet/off-MYC transgene of inducible Myc expression to mimic the effects of deregulated Myc on lacZ mutant levels in primary B cells. It demonstrated that deregulated Myc sensitizes B cells to undergo increased mutagenesis on treatment with 4-NQO. Although our studies with these model systems established a clear link between the natural history of pristane-induced PCT (inflammation and Myc) and elevated mutations in B cells, they did not suggest that the mutator phenotype of PCT was actually caused by inflammation or Myc. Some considerations leading to the alternative conclusion that the hypermutability of PCT is a complex phenotype that may be governed in part by obscure mutator genes in the tumor cells will be discussed in the following.

A brief one-day coinoculation of PECs with proliferating B cells in vitro resulted in a 3.7-fold increase in lacZ mutant levels in the B cells. The average increase in lacZ mutant levels in primary PCT was higher (5.4-fold) than in PEC-exposed B cells, yet the additional increase seemed negligible if one considers that the tumor cells accumulated mutations over an average period of 180 days (mean tumor onset). The steady, daily accumulation of 3.7-fold increases in MF over 180 days would lead, in theory, to a 666-fold increase in mutant levels in PCT, but this was obviously not the case. It thus appears that inflammatory cells have a much larger potential to cause mutations in B cells than is reflected by lacZ MF in plasma cell tumors. The great majority of B cells with mutations in lacZ may be eliminated in vivo, possibly by p53-dependent mechanisms of apoptosis, an important safeguard against Myc-driven B-cell tumors (56, 57). A recent study on MFs in pUR288 mice, deficient in the non-homologous end-joining pathway of DNA double-strand break repair (Ku80 null) but proficient for p53, supported the view that genomic integrity can be maintained in the face of increased mutagenesis, provided that the checkpoints of apoptosis are operational (58). The remarkable stability of MF during serial propagation of PCT also argues against the hypothesis that chronic inflammation is the main determinant of lacZ mutant levels in the tumors. MFs in transplanted PCT (42.3–47.0 \times 10^{-5}) and primary PCT (40.3 \times 10^{-5}) were essentially the same, even though the transplanted PCT had a significantly shorter exposure to the mutagenic environment of the pristane granuloma (35 ± 7 days) than the transplanted PCT (180 ± 45 days). The apparent stability of lacZ MF in serially transplanted PCT was strong evidence that the hypermutability of PCT is neither determined by a high mutation rate in PCT nor by persistent exogenous damage inflicted by inflammatory cells. The hypermutability may be transient, e.g., as a consequence of acute up-regulation of Myc after chromosomal translocation, or caused by intrinsic features of the tumor cell, such as mutator genes.

The overexpression of Myc in Tet/off-Myc-pUR288 bitransgenic B cells doubled 4-NQO-induced mutations compared with B cells without overexpressed Myc. The mutation-enhancing effect of Myc can be added to a growing body of evidence that deregulated Myc can function as a mutator gene (reviewed in Refs. 43, 44). Some mechanisms by which Myc induces genetic instability and increased mutagenesis are currently emerging (21, 22, 24, 26–28). However, our finding that overexpressed Myc in Tet/off-Myc-pUR288 B cells exposed to oxidative cell culture stress (59, 60) in the absence of 4-NQO was not accompanied by elevated mutagenesis indicated that deregulated Myc is not a mutator gene per se. In fact, the lacZ mutant levels in Myc-overexpressing B cells were lower than in the controls: 11.9 \times 10^{-5} versus 14.7 \times 10^{-5} after 3 h of cell culture and 17.2 \times 10^{-5} versus 20.0 \times 10^{-5} after 27 h. Lack of DNA damage was not the underlying reason, because normal B cells in cell culture (Myc off) showed a clear upward trend in lacZ MF over time: 9.66 \times 10^{-5} at 0 h, 14.7 \times 10^{-5} at 3 h, and 20.0 \times 10^{-5} at 27 h. These results indicated that the impact of deregulated Myc on MF in B cells is variable and dependent on the type and intensity of the mutagenic attack. The pro-mutagenic potential of Myc may be fully restrained under conditions of moderate oxidative stress, possibly because of Myc-dependent adaptive responses that involve the up-regulation of key components of oxidative stress control, such as thioredoxin peroxidase, glutathione transferase, and lactate dehydrogenase. Lactate dehydrogenase is clearly activated in mouse plasma cell tumors (Supplementary Fig. 23), leading to increased production of pyruvate, a scavenger of oxygen radicals (23, 61). The ability of Myc to positively regulate DNA repair genes (62), including NBS1 (63) involved in DNA double-strand break repair, may further contribute to harnessing the detrimental effects of Myc on the genome. These considerations suggest that although Myc may be involved in the PCT mutator phenotype, Myc is unlikely to be the sole cause for this phenotype.
In conclusion, mouse PCTs exhibit the increased mutability of a lacZ reporter gene, which is associated with, but presumably not caused by, chronic inflammation and activated Myc. The elevated lacZ mutant levels can be added to previously reported manifestations of genomic instability in PCTs, e.g., cytogenetic aberrations (64) including Myc-activating chromosomal translocations (65), activation of intracisternal A particles (66), and DNA mutations due to deficiencies in transcription-coupled nucleotide excision repair (67) and DNA double-strand break repair (53, 54). Thus far, none of these changes has been elucidated with regard to the underlying molecular mechanism, and the relationship to deregulated Myc has also remained unclear. On the other hand, it has been shown that Myc-dependent mouse B-cell and plasma cell tumors can be associated with different mutant phenotypes. For example, Burkitt-like lymphomas of α-MYC-pUR288 bitransgenic mice exhibited the same propensity to rearrangement mutations in lacZ as PCTs did, but demonstrated, in contrast to PCTs, a very small increase in lacZ MF. These findings illustrate the persistent difficulty of distinguishing mutant phenotypes associated with Myc from mutant phenotypes caused by Myc. Our newly developed model of mutation assessment in pUR288 transgenic mice and pUR288-Tet-off/MYC bitransgenic mice may be useful to elucidate this distinction.

ACKNOWLEDGMENTS

We thank Dr. Jan Vign, University of Texas at San Antonio, San Antonio, TX, for providing pUR288-transgenic B6 mice; Drs. Stephen M. Holland and Harry L. Malech, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, for the kind gift of p47phox null mice; Drs. Martin Döllé, University of Texas at San Antonio, and Michael Boerrigter, Leven, Inc., Bogart, GA, for advice on the pUR288/lacZ mutagenesis assay; the staff of the Laboratory of Genetics’ mouse facility, particularly Wendy DuBois and Lisa Craig, for help with the in vivo studies; Drs. Joong Su Kim and Kee Oh Chay, Laboratory of Genetics (NICI), for performing the Southern blots; Dr. Sung Sup Park and Alan Coleman, NCI, for fluorescence in situ hybridization mapping of the genomic integration site on Chr 3 of pUR288; Dr. Robert Sobol, National Institute of Environmental Health Sciences, NIH, Bethesda, MD, for providing the plasmid, pSV40-T Ag; Dr. Lynne Rockwood for reading the manuscript and making helpful editorial suggestions; and Drs. Michael Potter and Beverly Mock for supporting this study.

REFERENCES

5. Tomlinson, I., and Bodmer, W. Selection, the mutation rate and cancer: ensuring that the tail does not wag the dog. Nat. Med., 5: 11–12, 1999.
537


Moderate Hypermutability of a Transgenic lacZ Reporter Gene in Myc-Dependent Inflammation-Induced Plasma Cell Tumors in Mice


Cancer Res 2004;64:530-537.

Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/64/2/530

Supplementary Material Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2004/02/05/64.2.530.DC1

Cited articles This article cites 68 articles, 32 of which you can access for free at: http://cancerres.aacrjournals.org/content/64/2/530.full.html#ref-list-1

Citing articles This article has been cited by 2 HighWire-hosted articles. Access the articles at: /content/64/2/530.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.