Lack of Ecotropic Virus Involvement in Induction of Lymphomas in DBA/2J Mice by 7,12-Dimethylbenz(a)anthracene

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Received 13 April 1990/Accepted 28 June 1990

DBA/2 mice carry a single endogenous ecotropic murine leukemia provirus, *Emv-3*, that is replication defective because of a single nucleotide substitution in codon 3 of p15^{gag}. However, when weanling DBA/2 mice are treated percutaneously with 7,12-dimethylbenz(a)anthracene (DMBA), ecotropic virus replication is induced in almost all of the treated mice. Previous studies have shown that this induction results from DMBA-induced reverse mutations in codon 3 that allow efficient virus replication. In addition to ecotropic virus replication, DMBA also induces lymphomas in 100% of the treated mice. These results have raised the possibility that ecotropic virus replication is causally associated with the development of lymphomas in DBA/2 mice, perhaps via the insertional activation or mutation of cellular proto-oncogenes. To test this possibility, we compared lymphoma incidence after percutaneous DMBA treatment in DBA/2J-*d*⁺/*d*⁺ mice, which carry two copies of *Emv-3*, with lymphoma incidence in DBA/2J-*d*⁺18J/*d*⁺18J mice, which lost both copies of *Emv-3* by homologous recombination involving the long terminal repeat sequences. The results of this study conclusively demonstrated that *Emv-3* is not causally associated with the development of DMBA-induced lymphomas in DBA/2J mice. Interestingly, histopathological and molecular analyses of the lymphomas indicated that the majority of the lymphomas in both strains of mice were of the B-cell lineage. This was unanticipated, since the majority of chemically induced lymphomas in other inbred strains are thymic lymphomas, presumably of the T-cell lineage. Thus, DBA/2 mice appear to present a unique model system for the investigation of chemically induced B-cell lymphomas in mice.

Inbred mouse strains differ in their susceptibility to lymphoma induction by chemical carcinogens (14, 26). For example, some strains, such as AKR, RF/J, and DBA/2, show a high incidence of thymic lymphomas (66 to 100%) when they are treated percutaneously with 3-methylcholanthrene (MCA), while other strains, such as A, C3HeB/Fe, BALB/c, MA/J, and CBA/J, display a high incidence of skin tumors when they are treated with MCA (50 to 100%) (14). Mice that develop skin tumors develop few or no induced lymphomas, whereas strains that are susceptible to lymphomas develop few or no skin tumors. Percutaneous treatment of DBA/2 mice with 7,12-dimethylbenz(a)anthracene (DMBA) results in 100% incidence of lymphomas with short latency periods (30, 31).

In some cases, tumor susceptibility is strongly influenced by the genotype of the mouse strain at the *Ah* locus (42, 43). Mice that are homozygous or heterozygous for the dominant *Ah*^b allele (for example, C57BL/6 mice) have elevated levels of aryl hydrocarbon hydroxylase activity after carcinogen treatment and are susceptible to skin tumors (14). Mice that are homozygous for the recessive *Ah*^d allele (for example, DBA/2 mice) do not show aryl hydrocarbon hydroxylase inducibility and have a high incidence of lymphomas following MCA treatment. The results of bone marrow transplantation studies suggest that resistance to MCA-induced lymphomas is encoded by the bone marrow rather than the host environment (21). The specific mechanism of aryl hydrocarbon hydroxylase-mediated lymphoma resistance remains to be determined.

Endogenous retroviruses are often causally associated with the spontaneous development of murine lymphomas.

These viruses induce disease via the insertional activation or mutation of cellular proto-oncogenes (6), and *Myc* and *Pim-1* have been demonstrated to be frequent targets of viral activation in virus-induced T-cell lymphomas of mice (9, 10, 28, 36, 38, 46, 48). However, the role of endogenous retroviruses in chemically induced lymphomas is not totally clear. For example, retroviruses do not appear to be involved in the induction of chemically induced T-cell lymphomas in RF/J mice (5, 17), and neither somatically acquired proviruses nor virally induced rearrangements in cellular proto-oncogene loci, such as *Myc* and *Pim-1*, have been observed in RF/J lymphoma DNAs. In contrast, virally induced *Myc* and *Pim-1* rearrangements have been detected in chemically induced T-cell lymphomas of AKR mice (46). These results suggest that both virally and chemically activated cellular proto-oncogenes can cooperate to induce thymic lymphomas in AKR mice.

The results of previous studies (30, 31) suggested that an endogenous retrovirus may be causally associated with DMBA-induced lymphomagenesis in DBA/2 mice. Lymphomagenesis is preceded by increased ecotropic virus expression in the majority of mice. This virus expression can be detected as early as 2 weeks after the initiation of DMBA treatment. *Emv-3* is the only endogenous ecotropic provirus that is carried in the DBA/2 germ line and is replication defective because of an AT-to-CG substitution in codon 3 of p15^{gag} (8, 24). This single nucleotide change leads to the substitution of the usual glutamine residue by a proline residue. The presence of proline at this position prevents myristylation of the *gag* precursor polypeptide Pr65^{gag} and subsequent virus assembly. While some of the rapidity of lymphomagenesis observed in these mice is likely due to their genotype at the *Ah* locus (*Ah*^d or uninducible), insertional activation of cellular proto-oncogenes may be in-

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FIG. 1. Cumulative levels of lymphoma incidence or mortality for DMBA-treated DBA/2J- d^v/d^v and DBA/2J- d^{+18J}/d^{+18J} mice.

involved. We recently demonstrated that DMBA-induced ecotropic virus expression in DBA/2 mice results from DMBA-induced reverse mutations in codon 3 of *Emv-3* p15^{gag}. These reverse mutations in codon 3 lead to amino acid substitutions that allow efficient virus myristylation (27).

Previous studies have shown that the dilute (d^v) coat color mutation carried by several inbred strains and substrains, including DBA/2, was caused by spontaneous integration of *Emv-3* into the mouse germ line. The d^v mutation is unstable and reverts at a frequency of 4.5×10^{-6} revertants per gamete tested. Reversion is caused by virus excision via homologous recombination between the viral long terminal repeats (7, 22, 39). The use of DBA/2J mice that had lost the *Emv-3* provirus by homologous recombination (DBA/2J- d^{+18J}/d^{+18J}) as controls allowed us to unambiguously examine the role of ecotropic viruses in DMBA-induced lymphomas of DBA/2J mice.

To determine whether ecotropic viruses are involved in the induction of lymphomas in DBA/2 mice, we treated 25 DBA/2J- d^v/d^v and 19 DBA/2J- d^{+18J}/d^{+18J} mice with 50- μ l portions of a solution containing 2 mg of DMBA per ml on shaved backs twice weekly for 7 weeks (30). The mice were raised in our breeding colony at the National Cancer Institute Frederick Cancer Research Facility. DMBA (Eastman Kodak Co.) was purified on silicic acid (13). The DMBA-treated mice were subsequently aged and monitored for lymphoma incidence. Mice exhibiting any signs of lymphoma or other disease were euthanized by CO₂ inhalation, and tissues were removed for histopathological and molecular analyses of tumors. Sections from lymph nodes, lungs, livers, pancreases, spleens, stomachs, colons, hearts, kidneys, bladders, thymuses, thyroids, salivary glands, bones, and bone marrow were stained with hematoxylin and eosin and used to histologically classify the neoplasias by the system of Pattengale and Taylor (33).

Lymphoma incidence. The cumulative percentages of lymphoma incidence or mortality for each strain are shown in Fig. 1. The levels of lymphoma incidence or mortality were nearly identical for the two strains. A histological classification of the tumors examined in this study is shown in Table

TABLE 1. Histological classification of DMBA-induced DBA/2J lymphomas

Histological classification	No. of DBA/2J mice	
	d^v/d^v	d^{+18J}/d^{+18J}
Lymphoblastic lymphoma	5	5
Lymphocytic lymphoma	1	1
Follicular center cell lymphoma	6	8
Myeloid leukemia	2	0
Undifferentiated leukemia	1	0
Erythroleukemia	0	1
Unclassified lymphoma or leukemia ^a	2	2
No lymphoma or leukemia	6	1
Not done	2	1

^a Unclassified lymphomas or leukemias were tumors from four mice that were found dead and thus were impossible to type because of autolysis of the tissue samples.

1. No significant differences in tumor number or tumor type were found between the two strains. The average ages of tumor onset for the two strains were also similar (81.9 ± 20.0 days for DBA/2J- d^v/d^v mice and 81.3 ± 10.2 days for DBA/2J- d^{+18J}/d^{+18J} mice). Thus, contrary to our expectations, ecotropic viruses did not appear to play an essential role in DMBA-induced lymphomagenesis in DBA/2J mice. Consistent with this conclusion, no new somatically acquired ecotropic or mink cell focus-forming proviruses were detected in DMBA-induced DBA/2J lymphomas by Southern blot analysis (data not shown).

Wasting disease. The only potentially significant difference between the two strains was observed between days 30 and 55 after initiation of DMBA treatment. During this time, four DBA/2J- d^v/d^v mice died of a wasting disease. No lymphomas or other apparent causes of death were observed in these mice when they were subjected to histological analysis. In contrast, none of the DBA/2J- d^{+18J}/d^{+18J} mice showed any evidence of disease during this period. Because of the design of this experiment, we presume that the wasting disease was caused by *Emv-3* expression. However, this presumption remains to be confirmed.

Wasting diseases have also been reported to occur in chickens (37) and jungle fowl (47) that are exogenously infected with avian leukosis virus. In jungle fowl, the wasting disease has been shown to be associated with the development of viremia and the subsequent production of group-specific antibodies against the viral *env* gene (47). Although the sample size was small, our results raise the possibility that the wasting disease in our four mice may have been causally associated with *Emv-3* replication.

Histopathological classification of lymphomas. Most chemically induced lymphomas that have been characterized previously in mice are thymic lymphomas, presumably of the T-cell lineage (16, 17, 20, 46). This T-cell origin has been confirmed for MCA-induced RF/J thymic lymphomas by using cell surface markers that are specific for different lymphocyte cell lineages (17). DMBA-induced DBA/2J lymphomas have not been characterized previously according to lineage. Of the DBA/2J- d^v/d^v and DBA/2J- d^{+18J}/d^{+18J} lymphomas and leukemias that were analyzed histopathologically, the majority (26 of 34 lymphomas and leukemias) were classified as lymphoblastic, lymphocytic, or follicular center cell lymphomas (Table 1). Lymphoblastic and lymphocytic lymphomas represent a heterogeneous group of lymphomas and can originate from either T or B cells (29, 32). Follicular

TABLE 2. Immunoglobulin and T-cell receptor gene rearrangements in DMBA-induced DBA/2J lymphomas

Histological classification	No. of lymphomas:		
	T (IgH ⁺ , Igκ ⁺ , Tβ ⁺)	Pre-B (IgH ⁺ , Igκ ⁺ , Tβ ⁻)	B (IgH ⁺ , Igκ ⁺ , Tβ ⁻)
Lymphoblastic lymphoma	1	7	1
Lymphocytic lymphoma	1	0	0
Follicular center cell lymphoma	0	8	4
Unclassified lymphoma or leukemia	0	0	2

center cell lymphomas are generally of the B-cell lineage. Thus, the results of the histopathological analysis alone suggested that many of the DBA/2J lymphomas were of the B-cell lineage. Since no significant differences in lymphoma type were observed between strains, they were grouped together in the subsequent analyses.

Molecular classification of lymphomas. To confirm and extend the histopathological results, we analyzed 24 DBA/2J lymphoblastic, lymphocytic, follicular center cell, or unclassified lymphomas and leukemias for rearrangements in immunoglobulin heavy chain (IgH) and immunoglobulin kappa light chain (Igκ) genes, as well as T-cell receptor beta-chain (Tβ) genes. These rearrangements provide useful markers for assignment of cells to the B- and T-cell lineages (19, 29, 45) and can be used to assign tumors to these lineages when the histopathological diagnosis is ambiguous. Tissue samples from brains and lymphomatous organs (usually spleens and lymph nodes) were frozen at -70°C. DNA preparation, restriction endonuclease digestion, agarose gel electrophoresis, transfer of DNA to nylon filters, hybridization, washing, and autoradiography were performed as described by Jenkins et al. (23), with the modifications described by Mucenski et al. (29). The following DNA probes were used to screen the lymphomas: IgH (a *Bam*HI-*Eco*RI fragment containing J_H1 through J_H4), Igκ (an *Eco*RI-*Bgl*II fragment spanning J_κ1 through J_κ5) (44), and Tβ 1 and 2 (pUCJ1 and pUCJ2, respectively) (25). The results of this analysis are summarized in Table 2 and Fig. 2 and 3. Most of the tumors appeared to be monoclonal, based upon the number and intensity of rearranged fragments detected in tumors (Fig. 2 and 3). Two tumors contained Tβ rearrangements, indicating that they were of the T-cell lineage. However, the majority of the tumors contained rearrangements solely in IgH or in both IgH and Igκ genes, indicating that they were B-cell tumors. These results are consistent with the histopathological results and indicate that the majority of DMBA-induced DBA/2J lymphomas are of the B-cell lineage. Since the preponderance of chemically induced lymphomas in other mouse strains appear to be of T-cell origin, DMBA-induced DBA/2J lymphomas are a valuable addition to the model systems available for the study of chemically induced lymphomas.

Other potential mechanisms of lymphomagenesis. The lack of ecotropic virus involvement in the induction of DBA/2J lymphomas suggests that DMBA induces disease directly by altering the expression of cellular proto-oncogenes. Several cellular proto-oncogenes, including *K-ras*, *H-ras*, *N-ras*, *neu*, and *raf*, have been shown previously to be altered in chemically induced murine and rat tumors and are likely candidates for genes involved in DBA/2J disease (1, 2, 4, 11, 12, 15, 18, 34, 40, 41, 49, 50). The nature of the lesion(s) has

FIG. 2. Rearrangements in IgH (A) and Igκ (B) detected with J-region probes in DBA/2J-*d*⁺/*d*⁺ and DBA/2J-*d*⁺18J/*d*⁺18J B-cell lymphomas. Lanes M, *Hind*III-cleaved λ DNA; lanes N, lymph node DNA; lanes S, spleen DNA. Each lane represents a tumor from a different animal.

been shown to vary as a function of the chemical carcinogen used for tumor induction, which is consistent with the hypothesis that the carcinogen is directly responsible for inducing these lesions (4, 35, 49, 50). For example, CG-to-AT transversions were detected in the first position of *H-ras* codon 61 in hepatomas induced in B6C3F₁ mice by *N*-hydroxy-2-acetylaminofluorene, while AT-to-GC transversions were detected in the first position of *H-ras* codon 61 in 1'-hydroxy-2',3'-dehydroestradiol-induced hepatomas (49).

Activation of *H-ras* by an A-to-T transversion in the second position of codon 61 has been demonstrated previously in skin papillomas induced by DMBA treatment (3, 35). This substitution results in the creation of an *Xba*I site. We tested all of the DBA/2J lymphomas and leukemias for

FIG. 3. IgH (A), Igκ (B), and Tβ (C and D) gene rearrangements in two DBA/2J-*d*⁺/*d*⁺ T-cell lymphomas. The arrows indicate the positions of unrearranged germ line fragments. Lanes M, *Hind*III-cleaved λ DNA; lanes N, lymph node DNA; lanes T, thymus DNA. Each lane represents a tumor from a different animal.

the presence of this mutation by Southern blot analysis of *Xba*I-*Bam*HI-cleaved tumor DNA with an *Hras* probe. The results were negative for all of the tumors analyzed (data not shown).

Activating lesions in *Hras* have been shown to occur in both benign and malignant tumors, indicating that these lesions can be involved in all stages of tumor progression (2, 4). More than one activating lesion can also be identified in a single tumor DNA (12), which is consistent with a multistep model of tumor progression (16). Since DBA/2J mice represent a unique system for the study of chemically induced B-cell lymphomas, it will be interesting to compare the nature of the activating lesion(s) present in DMBA-induced B-cell lymphomas with the nature of the lesions present in chemically induced thymic lymphomas generated in other inbred mouse strains.

We thank Anthony Dipple for the gift of purified DMBA, Deborah Swing for technical assistance, and Xiao-Bei Liao, Monica Justice, Linda Siracusa, and Archibald Perkins for reviewing the manuscript. J.A.M. is a Leukemia Society of America Fellow.

This research was sponsored by the National Cancer Institute under contract NO1-CO-74101 with ABL.

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