THE GENETIC BASIS OF SUSCEPTIBILITY TO LEUKEMIA INDUCTION IN MICE
BY 3-METHYLCHOLANTHRENE APPLIED PERCUTANEOUSLY*

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In the course of earlier studies of the neoplastic effects of treatment with both 3-methylcholanthrene (MCA)[1] applied percutaneously and vaccinia virus inoculated intradermally in mice of various inbred strains (1, 2) we noted that the response to MCA painting, with or without vaccinia, was strain-specific. Mice of several strains developed a high incidence of skin tumors with little or no leukemia, whereas mice of other strains developed few or no skin tumors but showed a high incidence of thymic leukemia.

Susceptibility to local tumorigenesis resulting from topical or subcutaneous treatment with hydrocarbon carcinogens has been shown to be strongly influenced by the genotype of the mouse at the *Ah* locus (3, 4). This gene governs the expression of the enzyme system known as aryl hydrocarbon hydroxylase (AHH) (5). Mice homozygous or heterozygous for the dominant *Ah^b* allele show elevated levels of AHH activity in their tissues after treatment with any one of a number of hydrocarbon carcinogens, including MCA, whereas mice homozygous for the recessive *Ah^d* allele do not show this enzyme inducibility. Genetic experiments have indicated that AHH inducibility is associated with a high level of susceptibility to the induction of local tumors occurring at the subcutaneous site of MCA inoculation (6).

Leukemia induction by topical application of MCA or related hydrocarbons has been known for many years to occur in mice of some strains (7, 8), but the phenomenon has received little attention in recent times. From our studies it appears that this response is specifically seen in mice homozygous for the *Ah^d* allele, *i.e.*, AHH-noninducible.

Materials and Methods

Mice. Animals of various inbred mouse strains were obtained for these studies from The Jackson Laboratory, Bar Harbor, Maine. F_1 crosses of some of these strains were bred in our own colony; in designating these hybrids, the strain of the female parent has been placed first.

Treatment. Female mice were treated beginning at 10-12 wk of age. Most groups received

MCA painting without pretreatment; in these mice, the flanks and back were shaved and then painted daily for 5 days with a solution of 1 g MCA (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) in 100 ml benzene using a marten brush. Some mice received a pretreatment consisting of five daily subcutaneous injections in the groin of cortisone acetate (1 mg in 0.1 ml saline; Merck Sharp & Dohme Canada Ltd., Quebec, Canada); on the day of the last injection of cortisone, the flanks and back of the mice were shaved, and a single intradermal inoculation into the flank of 0.1 ml of vaccinia virus (Levaditi strain) suspension was administered. The vaccinia suspension was a 10% extract in saline of infected rabbit testes with an infective titer in rabbit skin of 10^7–10^8. MCA painting, as above, was commenced the day after vaccinia inoculation. Both treated and control mice were observed for tumors or other manifest disease for at least 500 days, if they survived that long, and they were sacrificed *in extremis* and autopsied.

AHH Determination. AHH activity was assayed by the method of Nebert and Gelboin (5) as modified by Thomas et al. (3). For enzyme induction mice received MCA (10 mg/ml in corn oil) intraperitoneally at a dose of 100 mg/kg body weight; control mice received corn oil. The mice were sacrificed 24 h later by cervical dislocation; their livers were removed, stored at −70°C and assayed for AHH activity 2–3 wk later. AHH activity in units/gram wet weight of liver was determined by the conversion of benzy(α)pyrene to hydroxylated benzy(α)pyrene equivalent in fluorescence (activation 396 nm, emission 522 nm) relative to 1 nmol of authentic 3-hydroxybenzo(α)pyrene (kindly provided by Dr. H. V. Gelboin, Chemistry Branch, National Cancer Institute, Bethesda, Md.) produced per 20 min incubation at 37°C. AHH inducibility was determined from the ratio of the mean activity in tissues from MCA-treated mice to that of control mice; mice were considered inducible when this ratio was higher than 1.3.

Results

Strain Distribution of Response to MCA Painting.   Of eight inbred strains carrying the Ah^d allele (AHH-noninducible) which were examined for their response to MCA painting, seven showed a significant incidence of lymphomas (Table I). In six of these strains the disease was mostly thymic leukemia, and in one (SJL) it was reticulum cell sarcoma. Only three strains showed a skin tumor response, and in each case this response was a weak one (two or fewer papillomas/mouse) occurring in only 11–20% of the mice tested.

Among these Ah^d strains only the AKR showed a significant incidence of spontaneous leukemia before 1 yr of age in the absence of MCA treatment (Fig. 1). In these mice the effect of the treatment was minimal, decreasing the mean age at which the disease was detected from 246 to 226 days. SJL mice also showed a high incidence of spontaneous lymphoma resembling Hodgkin's disease at a considerably later age than AKR mice, but in this case the occurrence of the disease was markedly accelerated by MCA painting: 76% of untreated mice developed the disease at a mean age of 381 days, whereas 84% of painted mice did so at a mean age of 267 days.

The leukemogenic effect of MCA painting was most pronounced in RF mice, which showed only a low and very late incidence of the spontaneous disease (Fig. 1). RF mice of the MCA-painted group showed a 93% incidence of the disease at a mean age of 209 days.

MCA-induced leukemia was also observed in four Ah^d strains which develop the spontaneous disease only occasionally in advanced old age. Two of these strains (DBA/1 and DBA/2) responded to MCA painting with a leukemia incidence of 66 and 52% and the other two (129 and ST/b) with an incidence of 33 and 20%.

The only Ah^d strain in which MCA-induced leukemia was not observed was

TABLE I

*Incidence of Skin Tumors and of Leukemia or Lymphoma in MCA-Painted
Mice of Different Inbred Strains According to Ah Genotype*

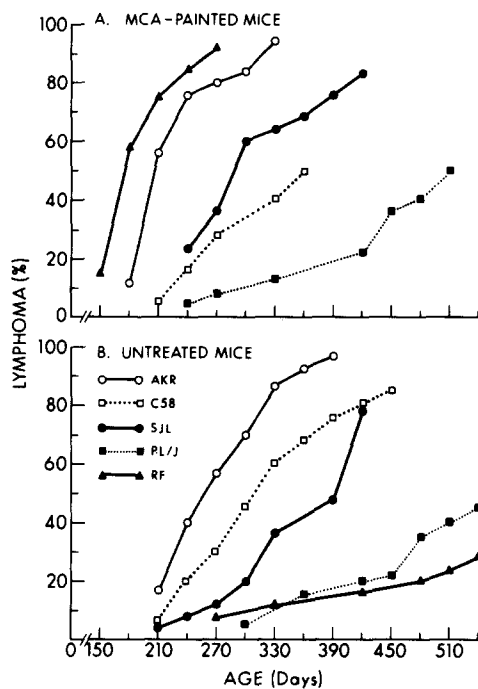| Mouse strain	Ah type	Number of mice	Percent of mice with:		Mean survival time
			Skin tumors	Lymphoma	
					days
AKR	d/d	25	0	100	146
RF/J	d/d	40	0	93	108
SJL	d/d	25	20	84	189
DBA/2	d/d	29	0	66	243
DBA/1	d/d	25	0	52	324
129	d/d	27	11	33	259
ST/b	d/d	30	0	20	310
SWR	d/d	20	15	0	307
C58	b/b	36	8	50	187
PL	b/b	22	5	50	340
A	b/b	20	50	0	350
C3HeB/Fe	b/b	25	60	0	275
BALB/c	b/b	40	80	0	240
MA/J	b/b	34	94	0	202
CBA/J	b/b	24	100	0	226

FIG. 1. Cumulative incidences of lymphoma in mice of different inbred strains (A) painted with MCA at 10–12 wk of age or (B) untreated. Strains AKR, SJL, and RF are Ah^d type (AHH noninducible; solid lines), and strains C58 and PL are Ah^b type (AHH inducible; broken lines).

the SWR, and these mice responded to the carcinogen with a high incidence of lung tumors.

Of the seven Ah^b (AHH-inducible) strains painted with MCA (Table I), five showed no leukemia but a high incidence (50-100%) of skin tumors. These tumors consisted of multiple early papillomas scattered at random over the painted skin of which one or more later evolved into malignant tumors.

Two Ah^b strains, C58 and PL, showed only a very low incidence (5-8%) of single small papillomas. These two strains showed a high incidence of leukemia, but this incidence of the disease was not significantly different from that in untreated controls (Fig. 1) and thus was not attributable to MCA painting. The low tumor response in these high-leukemic strains may have been due to the high levels of infectious, endogenous murine leukemia virus (MuLV) in their tissues, since, in low-MuLV strains, infection with exogenous MuLV is known to inhibit skin tumor induction by MCA (2, 9).

These results indicate that the strain distribution of the neoplastic response to MCA painting in inbred mice is correlated with the strain distribution of alleles at the Ah locus. The carcinogen induced a high incidence of leukemia or enhanced the occurrence of the spontaneous disease only in mice with the recessive Ah^d allele, whereas it induced a high incidence of skin tumors only in mice with the dominant Ah^b allele.

Response to MCA Painting in F_1 Crosses.   If, as the correlation among inbred mouse strains suggests, the Ah locus is indeed responsible for the observed polymorphism in the neoplastic response to MCA painting, then a similar correlation should be demonstrable among F_1 hybrids. Since the Ah^b allele is dominant in Ah^b/Ah^d heterozygotes with respect to AHH inducibility it should also be dominant with respect to its association with the skin tumor response to MCA. The data of Table II indicate that this was the case. In mice receiving MCA paintings alone (group MCA) the F_1 hybrids homozygous for the Ah^d allele showed high incidences of MCA-induced leukemia and developed no skin tumors; the Ah^b/Ah^d heterozygotes showed low incidences of leukemia, similar to those in the untreated group, and developed modest but significant incidences of skin tumors.

However, since the skin tumor response to MCA painting in the Ah^b/Ah^d heterozygotes was much weaker than that in the Ah^b parents (Table I), we resorted to a treatment shown in previous studies to enhance the skin tumor response to MCA without significantly enhancing the leukemic response (1). Thus, the hybrids were pretreated with cortisone, to render their skin responsive to the acute effects of vaccinia virus, and then with vaccinia inoculated intradermally before MCA painting (group C-V-MCA). The results confirm that Ah^b/Ah^d mice showing a relatively weak skin tumor response to MCA alone showed a considerably stronger skin tumor response after cortisone and vaccinia pretreatment without developing an increased incidence of leukemia. Pretreatment of Ah^d homozygotes, on the other hand, had no effect on their resistance to the skin tumors and had little or no influence on their leukemic response.

The occurrence of spontaneous leukemia in untreated mice of these F_1 crosses showed no correlation with their genotype at the Ah locus, as was the case in mice of the various inbred strains observed above. From the data in

TABLE II

Incidence of Skin Tumors and of Leukemia in Different F_1 Hybrid Mice Treated with Cortisone, Vaccinia Virus, and MCA (C-V-MCA), with MCA Alone or Untreated, According to Ah Genotype

Mice	Ah type	Treatment	Number of mice	Percent of mice with:		Mean age at death
				Leuke-mia	Skin tu-mors	
						days
(AKR × RF)F₁	d/d	None	33	33	0	>500
		MCA	40	80	0	325
		C-V-MCA	40	88	0	253
(AKR × DBA/2)F₁	d/d	None	50	22	0	>400
		MCA	27	78	0	315
		C-V-MCA	28	79	0	290
(DBA/2 × RF)F₁	d/d	None	50	8	0	>500
		MCA	27	81	0	297
		C-V-MCA	28	93	0	255
(C3H × AKR)F₁	b/d	None	27	22	0	>500
		MCA	20	20	10	458
		C-V-MCA	21	33	67	351
(C3H × RF)F₁	b/d	None	65	8	0	>500
		MCA	40	18	33	453
		C-V-MCA	40	20	90	368
(BALB/c × AKR)F₁	b/d	None	40	5	0	>500
		MCA	40	5	25	483
		C-V-MCA	40	0	85	380
(BALB/c × RF)F₁	b/d	None	50	0	0	>500
		MCA	40	10	60	388
		C-V-MCA	40	10	100	312

Table II, it is clear that the high and early incidence of the spontaneous disease of AKR mice was recessive in all crosses examined, whether the other parent was of the Ah^b (C3HeB, BALB/c) or Ah^d (RF, DBA/2) genotype. These and other unpublished observations in our laboratories indicate that many low-leukemic inbred strains carry one or more dominant genes capable of suppressing the leukemic phenotype of AKR mice, but that the Ah locus is not involved in this phenomenon.

The neoplastic response of these F_1 mice to MCA painting, on the other hand, does appear to be strongly influenced by their Ah type (Table II). F_1 mice homozygous for the Ah^d allele showed a 78-81% incidence of leukemia following treatment with MCA alone and a 79-93% incidence of the disease after combined treatment with cortisone, vaccinia and MCA. Ah^b/Ah^d hetero-zygotes, however, showed leukemia incidences of only 5-18% after treatment with MCA alone and of 0-33% after combined treatment with cortisone, vaccinia and MCA. No skin tumors were seen in MCA-painted Ah^d homozy-

gotes, whether pretreated with cortisone and vaccinia or not, whereas Ah^b/Ah^d heterozygotes showed incidences of 10–60% after MCA treatment and 67–100% after combination treatment.

Earlier studies (1) indicated that the capacity of vaccinia to enhance the skin tumor response to MCA painting appears to be correlated with its capacity to induce acute local ulcerative lesions in the skin in the absence of MCA painting. Susceptibility to these virus-induced lesions was noted to vary considerably in cortisone-pretreated mice of different inbred strains, BALB/c, C3HeB, A, and C58 mice being susceptible and DBA/1, DBA/2, B10.D2, and AKR mice being resistant to the lesions. By hindsight it is clear that this strain distribution is, with one exception, also that the Ah gene polymorphism, Ah^b mice (except B10.D2) being susceptible and Ah^d mice resistant to the lesions. This observation was borne out in the results of the present studies. In the F_1 crosses examined, Ah^b/Ah^d heterozygotes were susceptible to the vaccinia lesions, whereas Ah^d homozygotes were resistant. These results suggest that dermal susceptibility to vaccinia infection may also be strongly influenced by the Ah type of the host, although further genetic experiments will be required to verify this hypothesis. The occurrence of this acute response to the virus in the skin of Ah^b but not Ah^d mice of group C-V-MCA was probably the basis of the enhanced skin tumor response to MCA noted in the Ah^b mice.

The C58 × RF Cross. Of particular interest was the (C58 × RF) F_1 hybrid. This cross of a parental strain (C58) which shows a high and early incidence of spontaneous leukemia with another strain (RF) which shows a lower and much later incidence of the disease produced F_1 hybrids whose incidence closely resembled that of the low-leukemic parent (Fig. 2). This finding indicates that the RF genome includes one or more dominant genes capable of suppressing the high-leukemic phenotype of C58 mice. This suppressor gene may be the same one responsible for the similar phenomenon noted in the AKR × RF cross (Table II).

The strong susceptibility of mice of the RF strain to MCA-induced leukemia was also recessive in this cross (Fig. 2), since the parental C58 strain provided the dominant Ah^b allele which caused these F_1 hybrids to respond with skin tumors rather than leukemia. Thus this hybrid appears to possess different dominant genes protecting it from both the early spontaneous leukemia of the C58 parent and the similar disease induced by MCA painting in the RF parent. To further clarify these findings we investigated the leukemogenic effects of MCA painting in RF × (C58 × RF) F_1 backcross mice pretreated, like the hybrids, with cortisone and vaccinia. The leukemic response of these mice to the carcinogen (Fig. 2) was intermediate, in terms of the incidence and the incubation time of the disease, between that of the F_1 parent and that of the RF parent. This finding is compatible with the interpretation that a single dominant gene from the C58 parent is responsible for the apparent suppression in the hybrids of the MCA-induced leukemia from the RF parent.

Segregation among (C3H × RF) × RF Backcross Mice. Ultimately the proof that the Ah locus governs susceptibility to MCA-induced leukemia should come from observation of co-segregation of AHH noninducibility and of MCA-induced leukemia in an appropriate backcross population. Since the test for AHH inducibility may not be valid when performed on an animal in the
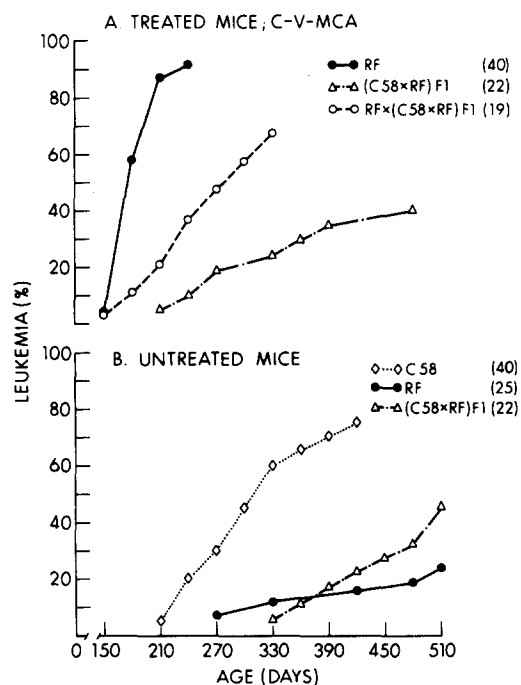
Fig. 2. Cumulative incidences of lymphoma in mice of the C58 and RF strains and their crosses (A) treated with cortisone, vaccinia virus, and MCA (group C-V-MCA) or (B) untreated.

terminal stages of MCA-induced leukemia, we carried out an indirect version of this experiment. Mice of the (C3H × RF) × RF backcross generation, which should consist of equal numbers of Ah^d homozygotes and Ah^b/Ah^d heterozygotes, were treated with cortisone, vaccinia, and MCA and separated randomly into two groups. Both groups were observed for the occurrence of skin papillomas which develop within 2 mo after MCA painting and very rarely thereafter. About 3 mo after treatment the mice of one group were tested individually for AHH inducibility, while the mice of the other group were observed for the development of MCA-induced leukemia which occurs only after a minimum latency of 3–4 mo after MCA painting. The results (Table III) indicate that AHH inducibility was directly correlated with high susceptibility to skin tumors, whereas leukemia was inversely correlated with skin tumor occurrence. These results offer a strong confirmation that susceptibility to leukemia induction by MCA painting is a phenotype conferred largely by the homozygous Ah^d genotype.

Discussion

Although it is by no means a new idea that chemical carcinogens can cause leukemia, the observation that skin painting of mice with MCA may sometimes lead to this disease (7, 8) has not received much attention in recent years. In the course of our studies of this phenomenon, it gradually became clear that MCA skin painting in mice of various strains usually induced either skin tumors or leukemia but rarely both. The two very different neoplastic responses

TABLE III

Occurrence of Skin Tumors (Papillomas) in Direct Relation to AHH Inducibility
(Group 1) and in Inverse Relation to the Occurrence of Leukemia (Group 2)
Among Mice of the (C3H × RF) × RF Backcross Generation

Group 1	AHH inducibility ratio*	Number of mice	Papillomas	
			Mice with tumors	Mean no. of tumors (range)
			%	
	1.5–2.3 (mean 1.9)	12	100	16.7 (6–33)
	0.7–1.2 (mean 1.0)	13	46	1.9 (1–6)

Group 2	Leukemic response	Number of mice	Papillomas	
			With tumors	Mean no. of tumors (range)
	Without leukemia	21	90	13.0 (2–32)
	With leukemia	16	25	5.0 (1–8)

* See Materials and Methods; AHH noninducible mice show a ratio of 1.0, whereas inducible mice show values significantly higher than 1.0.

appeared to be strain-specific and, by and large, mutually exclusive outcomes of the treatment. In crosses of skin tumor-responding and leukemia-responding strains, the skin tumor response emerged as the genetically dominant trait, albeit at a weaker level than in the corresponding parental strain.

In mice of segregating backcross generations to the leukemia responding strain the two types of MCA-induced neoplasms again appeared to be essentially mutually exclusive and to occur each in about half of the animals. Since it was known that susceptibility to local tumor induction by hydrocarbon carcinogens was strongly associated with the dominant Ah^b allele for AHH inducibility (6), we investigated the possibility that leukemia induction by the same chemicals was associated with the recessive Ah^d allele, and our present studies have confirmed this hypothesis.

Although it is not difficult to conceive that induction of the AHH enzyme system by MCA painting might play a role in the genesis of local tumors at the site of application, it is more problematic to understand how the same treatment might in the absence of AHH inducibility lead to thymic leukemia. In Ah^d homozygotes, does the chemical act directly on cells of the lymphatic system, or is its effect indirect? If the effect is direct, is it exerted upon lymphocytes which are present transiently in the painted skin and which return to the lymphoid organs, or is the chemical transported to these organs? In any case, it appears that the absence of AHH inducibility in Ah^d homozygotes is not a causal factor in MCA leukemogenesis, but rather a prerequisite for its occurrence.

Another set of unanswered questions concerns the possible role of endogenous MuLVs in these MCA-induced leukemias. While there is little direct evidence for viral involvement in MCA skin tumorigenesis, MuLV has proved to be associated with mouse leukemia under a wide variety of circumstances, including the spontaneously occurring disease which appears to be highly dependent

on the level of endogenous MuLV (10) as well as X-ray-induced (11, 12) and chemically induced (13-15) leukemia. Accordingly, it is tempting to speculate that the primary tumorigenic effect of MCA, which presumably occurs as a consequence of interaction between the carcinogen (or its metabolites) and cellular DNA, might be different in the two types of mice: disruption of cellular regulation by induction of somatic mutations in the case of skin tumorigenesis in Ah^b mice, and induction of endogenous MuLV expression in leukemogenesis in Ah^d mice.

In three of the Ah^d strains studied (129, ST, and SWR), MCA painting elicited few or no skin tumors, as expected from our working hypothesis, but also induced leukemia in fewer than 50% of the mice tested. If induction of endogenous MuLV expression is indeed a causal factor in MCA leukemogenesis, it is possible that these strains lack appropriate inducible endogenous MuLV genomes. In the case of strain 129 mice, for example, no endogenous ecotropic MuLV has been detected to date (16). Alternatively, appropriate MuLV genomes may be present but in a form not susceptible to induction by MCA.

Two of the Ah^b strains tested (C58 and PL/J) were strains which show relatively high incidences of spontaneous leukemia associated with a high level of endogenous MuLV expression in their tissues. MCA painting did not increase the incidence of leukemia in these mice (Fig. 1): indeed, painted C58 mice showed a lower incidence of leukemia than untreated controls, but this finding is attributable to the shortened mean survival of the painted mice as a result of their extremely severe and extensive skin ulcerative response to the carcinogen — a response also seen in other members of Little's C57-C58 family of inbred strains. However, the skin tumor response anticipated in MCA-painted C58 and PL/J mice on the basis of their Ah^b type proved to be extremely weak. It is likely that the high levels of MuLV observed in these animals interfered with the induction of skin tumors by MCA painting, since it has been shown that MuLV infection of low-MuLV mice inhibits the local neoplastic effects of MCA (2, 9).

MCA painting of AKR mice induced only a modest augmentation of the leukemic response in the form of a slightly earlier mean age of development of the disease by comparison with normal controls. This observation is presumably due to the fact that the genetic barriers to spontaneous leukemogenesis which exist in most Ah^d strains and which are overcome by MCA painting are missing in AKR mice so that the treatment is superfluous.

A correlation previously observed in mice of various inbred strains between skin susceptibility to MCA tumorigenesis, on the one hand, and skin susceptibility to vaccinia-induced ulcerative lesions, on the other (1) was again noted in mice of the F_1 crosses examined in the present studies. The findings suggest but do not prove that the virus-induced lesions occur specifically in Ah^b mice and that Ah^d homozygotes are resistant to them. These lesions result from extensive productive infection by the virus of target cells within the skin; in resistant mice infectious virus is detected at the site of inoculation in lower titers and for shorter periods of time than in susceptible hosts (1). Resistance to the lesions appears to operate at the level of the target cell rather than systemically, since skin from resistant parental strain mice retains its resistance when grafted onto a susceptible F_1 recipient (17). However, pretreatment

with cortisone is necessary for the development of the lesions even in susceptible mice. If the Ah genotype is indeed a factor in the occurrence of these lesions, it is possible that the effect is exerted at the level of susceptibility to the capacity of cortisone to render the target cells sensitive to virus infection.

Summary

Susceptibility to leukemia induction in mice by skin painting with 3-methylcholanthrene (MCA) is strain-specific, occurring only in strains relatively resistant to MCA-induced skin tumors. The Ah locus, which has a dominant allele (Ah^b) for inducibility of the aryl hydrocarbon hydroxylase (AHH) enzyme system and a recessive allele (Ah^d) for noninducibility, appears to be the major determinant of this trait. MCA-painted mice of strains and crosses carrying the Ah^b allele usually show a high incidence of skin tumors (papillomas which may evolve into malignant tumors) and little or no leukemia, whereas in mice homozygous for the Ah^d allele the treatment usually induces a high incidence of leukemia and few or no skin tumors. Among mice of a segregating backcross generation including both Ah^b/Ah^d heterozygotes and Ah^d homozygotes, the occurrence of skin tumors was correlated directly with AHH inducibility and inversely with the leukemic response. Mice of Ah^b strains with a high level of endogenous murine leukemia (MuLV) expression (C58, PL) show a much weaker skin tumor response than expected but no increase in leukemia incidence, and this observation tends to confirm the previous finding that MuLV infection of mice of low-MuLV strains results in reduced susceptibility to MCA tumorigenesis.

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