Mouse Mammary Tumor Virus-Induced Tumorigenesis in *sag* Transgenic Mice: a Laboratory Model of Natural Selection

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Transgenic mice that expressed the superantigen protein encoded in the C3H exogenous mouse mammary tumor virus long terminal repeat deleted their V β 14⁺ T cells during the shaping of their immune repertoire and showed no evidence of virus production in their mammary glands after infection by milk-borne C3H exogenous virus. However, they developed mammary gland tumors that had newly integrated copies of C3H exogenous virus, although the latency of tumor formation was much longer than in their nontransgenic littermates that retained their V β 14⁺ T cells. After four generations, infectious C3H virus was completely eliminated from the transgenic mouse pedigree. These data support the hypothesis that endogenous mouse mammary tumor proviruses are retained in the genome as protection against exogenous virus infection and subsequent tumorigenesis and show that there may be natural selection against the virus in vivo.

Mouse mammary tumor virus (MMTV) causes a high incidence of mammary tumors in females of strains susceptible to infection. MMTV virions are produced in the mammary glands of infected females and transferred via milk to suckling newborn pups. As adults, these neonatally infected females develop mammary gland adenocarcinomas with a latency period and incidence characteristic of their particular inbred strain. For example, virus-infected C3H/He (C3H/He MMTV⁺) inbred mice express high titers of MMTV in their milk, and approximately 90% of their offspring develop mammary tumors between the ages of 7 and 10 months (15). MMTV induces mammary gland tumors by acting as an insertional mutagen, activating cellular oncogenes. A number of novel oncogenes have been implicated in MMTV-induced mammary tumors (16).

In addition to the exogenously transmitted virus, endogenous proviral copies of MMTV have been detected in all common strains of laboratory mice (13). Different inbred strains contain distinct proviruses at various genomic loci (13). More than 20 different germinally transmitted *Mtv* loci have been described on the basis of molecular hybridization studies (13, 22). With the exception of *Mtv-1* (24) and *Mtv-2* (23), which are expressed as infectious viral particles in mammary glands, all of these proviruses are apparently nontumorigenic.

It has recently been shown that the open reading frame (ORF) protein encoded in the long terminal repeat of MMTV acts as a minor lymphocyte-stimulating antigen, or viral superantigen (Sag), causing the proliferation of T cells bearing specific V β chains of the T-cell receptor in vitro (3) and the deletion of the same T cells in vivo during shaping of the immune repertoire (2). Different MMTVs cause the deletion or stimulation of different cognate T cells, most likely due to a 10 to 15% variation in the amino acid sequence of the putative extracellular C-terminal domain of the Sag proteins (4, 7, 8, 17, 25, 26).

The identification of the MMTV ORF protein as a Sag led to the hypothesis that it played a role in the interaction of the virus with cells of the immune system (1, 3, 5). Previously, we established transgenic mouse strains in the C3H/HeN inbred background that expressed the C3H exogenous MMTV Sag

protein and deleted from their immune repertoire T cells bearing the V β 14 chain of the T-cell receptor (9). The transgenic mice that deleted the majority of their V β 14⁺ T cells showed no evidence of infection by exogenous C3H MMTV after suckling on viremic mothers. We hypothesized that inbred strains of mice that retained naturally occurring endogenous *Mtv* loci would have a selective advantage over animals not containing such proviruses, since mammary tumor incidence in the former would be reduced or abolished.

To test this hypothesis, we examined whether the *sag* transgene did indeed prevent MMTV-induced mammary gland tumors. Two types of MTV-ORF transgenic strains that expressed different levels of the transgene in the C3H/HeN inbred background were used in these experiments (9). Strains MTV-ORF 13, 16, and 35 expressed high levels of the *sag* transgene RNA and showed 80 to 90% deletion of their V β 14⁺ T cells in both the CD4⁺ and CD8⁺ populations as early as day 1 after birth. In contrast, strain MTV-ORF 19 mice, in which transgene expression was virtually undetectable by RNase protection analysis, showed only about 40 to 50% deletion of their V β 14⁺ T cells, and these T cells retained the ability to be stimulated by the C3H exogenous Sag protein.

The effect of the *sag* transgene on the susceptibility of C3H/HeN females to infection with milk-borne virus was tested. For this purpose, transgenic males of the three highly deleting strains (MTV-ORF 13, 16, and 35) and the low-expressing, low-deleting strain 19 were mated to C3H/HeN MMTV⁺ females, and F₁ offspring were produced (Fig. 1). Because the transgenic males were heterozygous for the transgene, both transgenic and nontransgenic progeny that had nursed on the same viremic mothers were generated from these crosses. Transgenic females and their nontransgenic littermates were force bred and observed for tumor formation and, by histoblot analysis of their offspring (9, 10), for virus production in milk.

We were not able to detect MMTV particles in the milk found in the stomachs of the offspring of the highly expressing MTV-ORF transgenic females that nursed on C3H/HeN MMTV⁺ nontransgenic mothers (Fig. 1, mouse B), even after their fifth pregnancy (Fig. 2, row A). In contrast, their nontransgenic littermates that nursed on the same mother (Fig. 1, mouse C) had large amounts of MMTV particles in their milk (Fig. 2, row B), even after their first pregnancy.

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FIG. 1. Pedigree of the MTV-ORF transgenic mice. Symbols: ○, nontransgenic female; □, nontransgenic male; ●, transgenic female; ■, transgenic male.

Although there was no evidence of viral infection in the MTV-ORF transgenic mice, they developed mammary gland tumors. In our experiments, the age at which 50% of the nontransgenic C3H/HeN females infected through milk developed tumors was about 225 days, and 90% of the females had tumors before 10 months (Fig. 3). In contrast, MTV-ORF transgenic mice that nursed on the same viremic mothers had mammary gland tumors with a much longer latency and lower incidence; 50% tumor incidence occurred at about 1 year, and at 10 months only 25% had developed mammary tumors (Fig. 3). Mice of the low-expressing MTV-ORF 19 strain, which retained Sag-reactive Vβ14⁺ T cells, had approximately the same incidence and latency of tumor development as the nontransgenic females (Fig. 3).

MMTV-induced mammary carcinomas contain one or more integrated MMTV proviruses in addition to their normal complement of endogenous viruses (6). The C3H/HeN inbred strain contains an endogenous provirus, *Mtv-1*, that is associated with late-developing mammary tumors in this strain (24). To determine whether the tumors that developed in the MTV-ORF transgenic mice were the result of C3H exogenous virus or the *Mtv-1* endogenous virus, DNA was isolated and examined by Southern blot analysis as previously described (21) for additional copies of exogenous or endogenous MMTV. Tumors from 14 transgenic and 11 nontransgenic mice were subjected to this analysis; shown here is the analysis of four transgenic mice and one nontransgenic mouse (Fig. 4A).

Tumor DNA was digested with *Pst*I and *Bgl*II and hybridized to the probe diagrammed in Fig. 4B, which allowed us to distinguish the newly integrated copies of exogenous MMTV (C3H) from the five endogenous MMTV proviruses (*Mtv-1*, *Mtv-6*, *Mtv-8*, *Mtv-11*, and *Mtv-14*) present in the C3H/HeN genome. The endogenous *Mtv* loci (except *Mtv-6*, which does not hybridize to this probe because it lacks the *gag*, *pol*, and *env* genes [13]) yielded fragments of 3.0 and 2.1 kb (Fig. 4A, lane 6). If the tumors were associated with newly integrated copies of *Mtv-1*, there would be an increase in hybridization to the 3.0- and 2.1-kb fragments. In contrast, any tumors associated

FIG. 2. MTV-ORF transgenic mice do not release viral particles in their milk. Histoblot analysis of the newborn offspring of MMTV-infected MTV-ORF transgenic (row A) and nontransgenic (row B) mice were done with an insert isolated from the plasmid Hybrid MMTV (21) as the hybridization probe. Data for offspring from the first, second, and fifth pregnancies are shown. The filters used for hybridization were stained with hematoxylin (shown above and below the autoradiographs).

with C3H exogenous virus would acquire new bands of 1.5 and 2.3 kb (Fig. 4B). All of the tumors, including those from the transgenic mice, had newly acquired copies of C3H exogenous MMTV and not *Mtv-1*, since each of them had the characteristic 2.3- and 1.5-kb bands (Fig. 4A; compare lanes 1 through 5 with lane 6).

Because the transgenic animals did develop C3H virus-associated mammary tumors, we tested whether a small amount of the virus was infecting the MTV-ORF transgenic mice that nursed on C3H/HeN MMTV⁺ nontransgenic females by examining whether their nontransgenic offspring were infected. To look at the transmission of virus, we performed the crosses diagrammed in Fig. 1. The F₁ transgenic and nontransgenic offspring of MTV-ORF transgenic mice that nursed on C3H/HeN MMTV⁺ mothers were mated with nontransgenic C3H/HeN males to produce pedigrees. Females generated from these crosses were force bred, and RNA from lactating mammary glands was isolated after the first, second, and fifth pregnancies and subjected to RNase protection analysis, as described previously (9).

Figure 5 represents the results of such an experiment. Transcripts from endogenous or exogenous virus protect a 270-nucleotide fragment containing sequences corresponding to the 5' end of the viral RNA, while the 5' end of the

FIG. 3. Mammary gland tumor incidence in MTV-ORF and non-transgenic mice infected with milk-borne virus. Tumor incidence was monitored by weekly palpation of animals, and mice bearing tumors were sacrificed. Portions of the tumors were saved as frozen tissue and in formaldehyde for histopathological analysis. The numbers of mice used in this analysis were 34 nontransgenic females; 10 MTV-ORF 19 (VB14⁺) transgenic females; and 30 MTV-ORF 13, 16, and 35 (VB14⁻) transgenic females.

transgene RNA protects a 101-nucleotide fragment. Transcripts from endogenous and exogenous virus, as well as from the transgene, protect a 115-nucleotide fragment corresponding to the 3' end of the RNA. After the first pregnancy, the F₂ nontransgenic offspring of transgenic MTV-ORF females that nursed on viremic mothers (Fig. 1, mouse D) did not show any accumulation of the viral RNA (Fig. 5, lane 2) that is characteristic of infection with C3H exogenous MMTV. Similarly, the MTV-ORF transgenic mother of this animal (Fig. 1, mouse B) showed no evidence of C3H exogenous viral RNA production in the lactating mammary gland, even after her fifth pregnancy (Fig. 5, lane 1). In contrast, RNA isolated from the lactating mammary gland of age-matched, nontransgenic C3H/He MMTV⁺ females (Fig. 1, mouse E) showed acquisition of exogenous virus after the first pregnancy (Fig. 5, lanes 3 and 4). The level of infection of the mammary gland and the quantity

FIG. 5. Transmission of MMTV by the MTV-ORF transgenic mice. RNAase T₁ protection analysis of RNA isolated from the lactating mammary glands of mice from the pedigree shown in Fig. 1. The labeled probe (p) used in these assays was described previously (9). There is some heterogeneity in the size of the fragments protected by the endogenous genes because of sequence heterology in their long terminal repeats, relative to the probe. Protected fragments are e₁, a 270-nucleotide fragment corresponding to the 5' end of viral RNA; t₂, a 101-nucleotide fragment corresponding to the 5' end of the transgene RNA; and t + e, a 115-nucleotide fragment corresponding to the 3' end of the transgene and viral RNA. In addition, there is a transgene-specific fragment (t₁), resulting from readthrough transcription of the tandemly linked transgene copies. The bands marked "x" correspond to exogenous virus-specific fragments that result from minor alternate termination sites. RNA was isolated from the mammary glands of an MTV-ORF 16 transgenic female (Fig. 1, mouse B) after her fifth pregnancy (lane 1); F₂ nontransgenic offspring of the MTV-ORF female (Fig. 1, mouse D) after the first (lane 2) and second (lanes 5 and 6) pregnancies; and offspring of a nontransgenic female (Fig. 1, mouse E) after the first (lanes 3 and 4) and second (lane 7) pregnancies. Lane 8 corresponds to yeast tRNA, used as a negative control. The bottom panel shows a Northern blot containing 10 µg of each RNA, hybridized to a mouse cytoskeletal β-actin probe.

FIG. 4. Detection of exogenous MMTV proviral DNA in mammary gland tumors. (A) High-molecular-weight DNA from tumors that developed in four MTV-ORF transgenic mice (lanes 1, 2, 4, and 5) and from a tumor from a nontransgenic infected C3H/HeN female (lane 3) and spleen DNA from an uninfected C3H/HeN mouse (lane 6) were digested with *Pst*I and *Bgl*II and subjected to Southern blot analysis with the hybridization probe depicted in panel B. (B) Map of the endogenous proviruses present in C3H/HeN mice, C3H exogenous virus, and the hybrid MMTV plasmid (21) from which the probe was derived. The presence of the 2.3- and 1.5-kb fragments is characteristic of newly integrated copies of exogenous virus. P, *Pst*I; B, *Bgl*II; X, *Xho*I.

TABLE 1. Exogenous MMTV is lost from the MTV-ORF transgenic pedigree

Mouse ^a	Generation	% of CD4 ⁺ T cells bearing Vβ14 ^b
C3H/HeN MMTV ⁻		8.2 ± 0.20
A		1.9 ± 0.30 (23)
B	F ₁	1.2 ± 0.20 (15)
D	F ₂	2.0 ± 0.50 (24)
E	F ₂	1.9 ± 0.05 (23)
F	F ₃	4.3 ± 0.10 (52)
G	F ₃	2.1 ± 0.05 (26)
H	F ₅	8.7 ± 0.30 (106)
I	F ₅	8.1 ± 0.05 (99)
J	F ₅	2.3 ± 0.30 (28)

^a The letters A through J correspond to the pedigree shown in Fig. 1.

^b T cells were isolated from the blood of the mice indicated, stained with antibodies against CD4 and Vβ14, and analyzed by fluorescence-activated cell sorter analysis with an electronically programmable individual cell sorter (Coulter Electronics Inc.), as previously described (9). Values are means for three to five mice, ± standard errors of the mean. All of the mice analyzed were 5 months old. Numbers in parentheses are levels of T cells present, shown as percentages of the level in normal C3H/HeN MMTV⁻ mice.

of virus produced by the nontransgenic mice increased with age and with the number of pregnancies (Fig. 5; compare lanes 3 and 4 with lane 7). After the second pregnancy, exogenous virus infection of the nontransgenic offspring of the MTV-ORF transgenic mice (Fig. 1, mouse D) also became apparent (Fig. 5, lanes 5 and 6). Nevertheless, virus accumulation was much lower than in nontransgenic, age- and pregnancy-matched C3H/He MMTV⁺ females (Fig. 5; compare lanes 5 and 6 with lane 7).

These results showed that exogenous MMTV was transmitted by transgenic MTV-ORF mice, but less efficiently than by nontransgenic C3H/HeN MMTV⁺ mice. As a result, passage of the virus through multiple generations of transgenic mice should decrease the total number of infectious viral particles and could result in the loss of virus in the lineage. To determine whether exogenous C3H MMTV was lost in the transgenic mouse pedigree, we measured the percentage of cognate Vβ14⁺ T cells in the immune repertoire of the nontransgenic offspring of different generations of MTV-ORF transgenic mice that nursed on viremic mothers (Fig. 1). Mice infected neonatally with exogenous C3H MMTV show a slow deletion of peripheral and thymic Vβ14-bearing T cells that is complete by the age of 5 months (12). If the MTV-ORF transgenic mice were infected with virus, their nontransgenic offspring should delete their Vβ14⁺ T cells, and the extent of this deletion should be proportional to the amount of virus they received.

In the F₂ generation, virus was transmitted from MTV-ORF mothers to their nontransgenic offspring, since the offspring showed a large deletion of their Vβ14⁺ and CD4⁺ T cells (Table 1, mouse D). By the F₃ generation, much less virus was transmitted in the transgenic lineage, since the extent of deletion of these T cells in 5-month-old nontransgenic offspring was 50% of that seen in the F₂ or F₃ nontransgenic mice (Table 1; compare mouse F with mouse D, E, or G). By the F₅ generation, there was no deletion of Vβ14⁺ T cells in nontransgenic offspring in the MTV-ORF lineage (Table 1; compare mice H, I, and J). The absence of cognate T-cell deletion in the F₅ generation from both transgenic and nontransgenic offspring of the F₄ mice (I and H) suggests that virus was completely eliminated by the F₄ generation.

These data indicate that the high-expressing MTV-ORF

transgenic mice were not completely protected against exogenous MMTV infection, since they still developed mammary tumors. These late tumors may occur because there is incomplete deletion of Vβ14⁺ T cells that can be stimulated by exogenous MMTV in the MTV-ORF transgenic mice. Since the total number of cells capable of being stimulated is low in the MTV-ORF mice in comparison with nontransgenic mice, less virus is acquired and hence the latency of tumor incidence is increased.

It took four generations to completely eliminate exogenous virus from the transgenic lineages. However, even partial protection against viral infection should confer a selective advantage. It has been shown that MMTV-infected C3H breeding females have an average life span of only 280 days in comparison with virus-free females, which have a life span of 492 days (19). In our study, the majority of tumors that developed in the virus-infected MTV-ORF mice had a latency of >1 year, thus increasing their reproductive life span. The occurrence of mammary tumors after 1 year would have an even smaller effect on the reproductive life span of mice in the wild, since most feral mice do not live that long (20). The delay in the onset of tumorigenesis should result in a higher breeding capacity in the mice that retain such endogenous *sag* genes. Thus, the preservation of the *sag* gene in the mouse genome could provide a selective advantage by decreasing mammary tumor incidence.

A naturally occurring example of this selection may have appeared in the MA/MyJ strain, which is mammary tumor free (14). This substrain was derived from a single female of the high-tumor-incidence MA strain (no longer in existence) that did not develop mammary gland tumors. The MA/MyJ strain carries a novel MMTV provirus, designated *Mtv-43* (18), that is not present in other inbred strains, implying that an infectious virus recently integrated into the germ line. That an infectious MMTV (MMTV-SW) that causes the deletion of the same cognate T cells as *Mtv-43* was recently isolated supports the idea that this novel endogenous virus in MA/MyJ mice protected its ancestor from infection (11).

In contrast to almost all known endogenous MMTV loci, which retain both *env* and *sag* sequences, the MTV-ORF transgene contains only *Sag* coding sequences. We have preliminary evidence that the presence of both the *env* and *sag* genes in transgenic mice results in better presentation to and more complete elimination of cognate T cells. Thus, the retention in the genome of complete copies of MMTV may better protect mice against viral infection. Nonetheless, even deleted proviruses, provided that they are still capable of coding for the *Sag* protein, may be retained in the genome as an antiviral defense.

We thank Paul Wright for expert technical assistance; A. Chervon-sky, J. Dudley and the members of our laboratory for helpful discussions; and G. Shackelford for the Hybrid MMTV plasmid. We gratefully acknowledge the expertise and equipment provided by the Flow Cytometry Laboratory of the Research Resources Center of the University of Illinois at Chicago.

This work was supported by a Cancer Research Institute postdoctoral fellowship to T.V.G. and by Public Health Service grant CA45954 from the National Institutes of Health.

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