Brief Definitive Report

INTERACTION OF THE HETEROZYGOUS NUDE GENE WITH THE ASPLENIA TRAIT IN MAMMARY TUMORIGENESIS

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BALB/cCrgl mice are characterized by a low incidence of spontaneous mammary tumors (SMT). Exogenous mouse mammary tumor virus (MMTV) is not found in this murine strain. However, these mice contain one subgenomic-size and two genomic-size MMTV proviruses (1). As previously reported (2), we have developed a colony of immunodeficient BALB/c mice by mating asplenic females with nude males that have been reconstituted with a congenic thymocyte intraperitoneal suspension equivalent to half a normal thymus. With these crossings, we have produced female breeders heterozygous for both nude and asplenia traits. Here, we present evidence indicating that the heterozygous nude trait increases the incidence of SMT. Furthermore, the simultaneous presence of the nude gene and the asplenia trait results in a very high percentage of SMT in BALB/c female breeders. Restriction endonuclease patterns of the liver DNA from normal and immunodeficient mice appear to be identical, with respect to the MMTV provirus copy number and organization. These results suggest that the increased tumorigenicity observed is due to a synergistic action of spleen agenesis with the heterozygous nude condition, during the latency period or at the host immune level.

Materials and Methods

Mice. The description of the development of the nude and asplenic colonies has been previously reported (2). The maintenance of the combined asplenia and heterozygous nude genes in BALB/c mice is accomplished by breeding *nu/nu* males with the asplenic females heterozygous for both the *Dh* and *nu* genes (*nu*/+, *Dh*/+). The progeny of such matings produces BALB/c offspring with four different phenotypes segregating equally among: *nu*/+ and +/+, of normal phenotype; *nu*/+ and *Dh*/+, characterized by a white coat, skeletal anomalies of the hind limbs, and spleen agenesis; *nu/nu* and +/+, nude (hairless) athymic; and *nu/nu* and *Dh*/+, the lasat mouse, with the combined characteristics of athymia, asplenia, hairlessness, and anomalous hind legs.

The BALB/c background of the various genotypes discussed above has been ascertained by grafting experiments. Skin grafts from mice of +/+, *Dh*/+; *nu*/+, +/+; or *nu*/+, *Dh*/+ genotypes were implanted in 2-mo-old BALB/c mice of +/+, +/+ background.

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The transplanted mice did not reject the skin grafts, indicating that the genetic makeup of the immunodeficient mice is BALB/c.

Nucleic Acid Isolation, Restriction Endonuclease Digestion, and Southern Blotting. DNA isolation, restriction endonuclease digestion, agarose gel electrophoresis, nucleic acid transfer to nitrocellulose, and filter hybridization was performed as previously described (3). α-[^{32}P]-labeled MMTV DNA complementary to 70 S MMTV RNA was prepared as previously reported (4).

Results

Female *nu*/+, *Dh*/+ breeder BALB/c mice develop a high number of SMT from an early age. On initial detection, tumors are ~0.5 cm in diameter. They grow rapidly, causing death within 30–45 d. We began a study to compare the relative incidences of SMT in a large number of BALB/c mice of various genotypes relating to the *nu* and *Dh* genes. Table I summarizes the results of these analyses. Of 492 normal BALB/c breeders in our colony, only four animals developed SMT. Introduction of the heterozygous *Dh* gene in BALB/c mice resulted in a small increase of the overall SMT incidence. BALB/c breeders heterozygous for the *nu* gene had a greater number of SMT before 19 mo. This incidence (23.8%) is significantly ($P < 0.01$) higher than that of the parent BALB/c strain without the nude gene (0.8%), or with the heterozygous *Dh* gene (5.8%). The asplenic female breeders of genotype *nu*/+, *Dh*/+ have the highest incidence of SMT (57.9%) before 19 mo of age. SMT were observed in neither *nu*/+, +/+ nor in *nu*/+, *Dh*/+ female virgins up to 16 mo of age. The generally shorter lifespan and difficulty of breeding female mice homozygous for the *nu* gene has precluded an adequate comparison of the SMT incidence in BALB/c of the *nu*/*nu*, +/+ genotype. Likewise, the *nu*/*nu*, *Dh*/+ (lasat) mice live only for 3–4 mo, and are not capable of successful matings.

The age distribution and percentages of SMT are presented in Fig. 1. Immunodeficient BALB/c breeders (*nu*/+, *Dh*/+) develop SMT as early as 5 mo of age. The maximum incidence occurred between 14 and 19 mo, but high levels of SMT were observed as early as 10 mo of age (21%). Very few BALB/c of the *nu*/+, +/+ genotype developed tumors during the first 15 mo (0.2–6.1%). However, at 19 mo, an incidence of 30.6% could be observed within the remaining 101 breeders of the original 507 experimental animals. These mice usually had one mammary tumor, while the *nu*/+, *Dh*/+ siblings often had from two to four SMT.

The germinally-transmitted MMTV proviral DNA was analyzed by restriction endonuclease mapping to determine if the BALB/c immunodeficient mice have

TABLE I

Incidence of SMT in Inbred BALB/c Mice

BALB/c genotype	Age at tumor appearance	Total number of mice	Mice with SMT	Overall incidence of SMT
	mo			%
+/+, +/+	≤20	492	4	0.8
+/+, *Dh*/+*	≤17	137	8	5.8
nu/+‡, +/+	5–19	507	133	23.8
nu/+, *Dh*/+	5–19	615	351	57.9

* *Dh*/+: heterozygous for dominant hemimelia.

‡ *nu*/+: heterozygous for athymia.

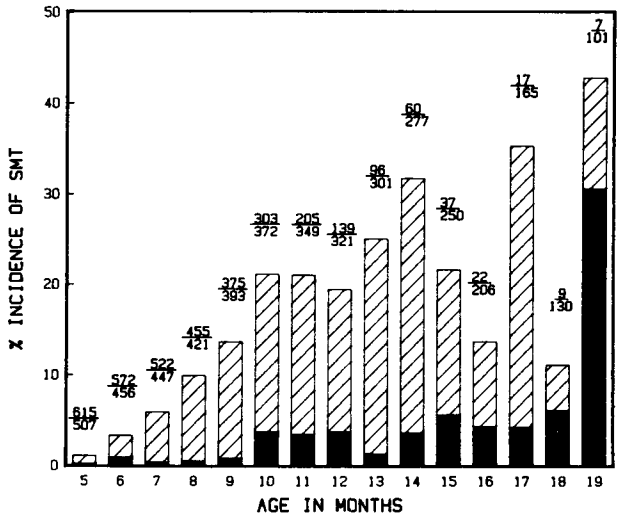

FIGURE 1. Comparison of the incidence and age distribution of SMT in BALB/c mice heterozygous for the nude gene (*nu*/+, +/+) (■), with that of mice heterozygous for both the *nu* and *Dh* genes (*nu*/+, *Dh*/+) (▨). The total number of mice of the *nu*/+, *Dh*/+ genotype is shown on the top of each column as the numerator, while the total number of mice of the *nu*/+, +/+ genotype is shown as the denominator.

a germ line complement of MMTV DNA identical to the characteristic BALB/c complement (1), or if they are characterized by an additional or altered MMTV germline complement. In addition, since the immunodeficient genotypes, *nu* and *Dh*, had been crossed into BALB/c mice from strain backgrounds that included C3H/He, C57BL/10J, and CBA/CaJ, MMTV maps from these strains are included for comparison. BALB/cCrgl, C3H/HeJ, C57BL/10J, and CBA/CaJ liver DNA were digested with Eco RI, Bam HI, or Pst I, and restriction fragments containing MMTV DNA were identified by molecular hybridization with an α-[^{32}P]-probe complementary to 70 S MMTV RNA (4, and Fig. 2). The results demonstrate that MMTV sequences in BALB/c DNA are distinguished from C3H/HeJ and C57BL/10J by Eco RI (Fig. 2) and Hind III (data not shown) digestions, and from CBA/CaJ by Eco RI and Pst I digestions (Fig. 2). Using these enzymes, at least two unique-size MMTV DNA fragments can distinguish between BALB/c DNA and MMTV sequences present in C3H/HeJ, C57BL/10J, and CBA/CaJ genomes. Restriction digestions with Eco RI, Bam HI, Pst I (Fig. 2), and Hind III (data not shown) demonstrated an identical complement of MMTV sequences between BALB/cCrgl DNA (Fig. 2, lanes *1,6*, and *11*) and liver DNA of a *nu*/+, *Dh*/+ mouse (Fig. 2, lanes *5*, *10*, and *15*). Therefore, the immunodeficient BALB/c *nu*/+, *Dh*/+ strain, as well as the *nu*/+, +/+; *nu*/*nu*, +/+; +/+, *Dh*/+, and *nu*/*nu*, *Dh*/+ (data not shown) genotypes contained a germline complement of MMTV DNA identical to the BALB/cCrgl +/+, +/+ strain, which has a low incidence of mammary tumors. That is, all of these immunodeficient BALB/c mice contain a subgenomic MMTV provirus, Unit I or mtv-6, and two genomic MMTV proviruses, Units II and III, or mtv-8 and mtv-9, respectively (1), that have previously (3, 5) been characterized in detail by restriction mapping, and molecular hybridization with cloned subgenomic

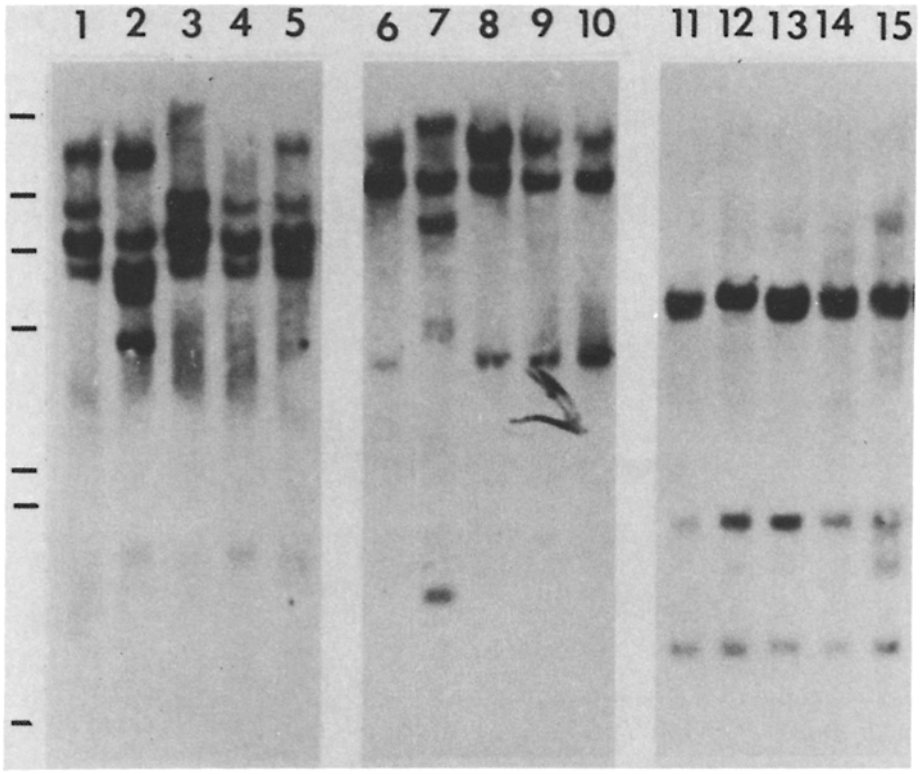

FIGURE 2. MMTV proviral DNA in inbred and immunodeficient mouse strains. Total cellular DNA was isolated from liver nuclei of 2-mo-old BALB/cCrgl (lanes *1*, *6*, and *11*), C3H/HeJ (lanes *2*, *7*, and *12*), C57BL/10J (lanes *3*, *8*, and *13*), CBA/CaJ (lanes *4*, *9*, and *14*), and BALB/c *nu/nu*, *Dh*/+ mice (lanes *5*, *10*, and *15*). 10 μg of DNA were digested with Eco RI, lanes *1–5*; Bam HI, lanes *6–10*; or Pst I, lanes *11–15*. Following electrophoresis and transfer, the filter was hybridized with α-[^{32}P]MMTV–complementary DNA. Autoradiography was for 27 h. Phage γ DNA digested with Hind III produced molecular weight references, indicated in the left margin, of 21.8, 9.5, 6.8, 4.3, 2.3, 2.0, and 0.6 kbasepairs.

MMTV probes. Importantly, these data demonstrate that mammary tumors in the immunodeficient BALB/c mice cannot be due to the presence of the C3Hf MMTV Unit V (mtv-1), which is responsible for late mammary tumors in C3Hf strains (6, 7).

Discussion

Our studies demonstrate that the coexistence of the *nu* and *Dh* genes in heterozygous form in female breeders of the BALB/c strain is associated with an early and dramatic increase in the incidence of SMT. No such increase in SMT could be observed in animals heterozygous for the *Dh* gene alone. However, a higher rate of SMT can be detected in female BALB/c breeders heterozygous for the nude gene. This is the first demonstration that the *nu*/+ genotype, as opposed to the *nu/nu* genotype, has any important effect on the host. This finding suggests the necessity for caution in interpreting experiments in which *nu*/+ mice serve as controls for an experimental *nu/nu* group.

A possible reason for the susceptibility of these mice to mammary tumorigenesis could be genomic acquisition of a provirus from an exogenous source. The

origin of our BALB/c immunodeficient mice includes the C3H background, where a mutation for dominant hemimelia was discovered by Searle (8) among a breeding stock of luxate mice. The *Dh* mutant is inherited as an autosomal dominant trait characterized by abnormalities that include skeletal anomalies (mainly of the hind limbs), and visceral alterations, in addition to splenic agenesis. Visceral defects in homozygous (*Dh/Dh*) mice result in death within a few days of birth. Asplenic BALB/c mice were developed by mating female F_1 hybrids of C57BL/6J-$A^{w\text{-}j}$ (*Dh*/+) female × CBA/Ca (+/+) male for 10 generations. These animals are designated B6CBAF_1-*Dh* (N10). The nude gene, discovered by Flanagan in 1966 (9), is inherited as an autosomal recessive gene and is characterized by congenital athymia in homozygous mice (10). The mice from which our colony originated were maintained on a BALB/c background for several generations. Thus, the origin of our immunodeficient mice includes, in addition to the BALB/c genotype, the possible proviral contributions from the low-SMT-incidence C57BL/10J and CBA/CaJ strains, and from the high-SMT-incidence C3H/H3 strain. However, the results of our restriction endonuclease MMTV mapping showed unequivocally that the germline MMTV sequences in all four classes of immunodeficient mice is identical to that of the BALB/c strain, and therefore rule out the possibility of perpetuation of an extra MMTV provirus that may have been acquired during the development of our animal colony.

Our studies suggest that the *nu* gene, in the heterozygous state, may influence mammary tumorigenesis in BALB/c mice, by shortening the latency period of oncogenesis, or by some other mechanism, such as altering the host immune response against the tumor. If the latter is the case, the observed effects may have a bearing in the controversial issue of "immune surveillance." Most investigators who have analyzed the incidence of spontaneous tumors in nude mice have used animals in which the nude gene has been backcrossed to the desired background only two or three times. According to Festing (11), five backcrosses are required in order to obtain a coefficient of inbreeding of >95%. Our nude mouse colony has had more than 20 generations of inbreeding. Therefore, effects of other loci on the SMT of *nu*/+, +/+ are unlikely. In addition, a high incidence of SMT in *nu*/+, +/+ mice are observed in female breeders up to 19 mo of age, which is consistent with the requirement for hormonally regulated mammary gland development and differentiation for mammary tumorigenesis. The *Dh* gene per se has only a modest effect on the overall incidence of SMT. However, it appears that the lack of spleen results in a syngergistic action with the heterozygous nude gene on SMT incidence in the BALB/c strain. Since spleen agenesis has been associated with alterations of the immune system, we hypothesize that the role of the *Dh* gene in our model system is one of amplification of the effect of the *nu*/+ genotype, at the level of host–tumor interactions.

Summary

The BALB/c mouse strain has been shown to contain endogenous mouse mammary tumor virus (MMTV) proviral sequences. However, no exogenous MMTV particles have been detected in their tissues. Female BALB/c mice from our colonies exhibit a very low incidence of spontaneous mammary tumors (SMT); <1% at up to 20 mo of age. Immunodeficient BALB/c mice heterozygous

for the nude gene (*nu*/+, +/+), for the dominant hemimelia gene associated with asplenia (+/+, *Dh*/+), or for both traits (*nu*/+, *Dh*/+) have been examined for SMT incidence and the presence of MMTV proviruses. Based on restriction digestion with Eco RI, Bam HI, and Pst I, the immunodeficient mice have an MMTV provirus copy number and organization identical to the BALB/cCrgl strain. This MMTV DNA pattern is distinct from the MMTV proviruses in C3H/He, C57BL/6J and CBA/CaJ mice, which were parental strains of the immunodeficient mutants. Normal female BALB/c or BALB/c heterozygous for the asplenic trait do not develop significant numbers of SMT at up to 19 mo of age. In contrast, an incidence of 23.8% and 57.7% SMT was observed in BALB/c *nu*/+ heterozygotes, and in BALB/c *nu*/+, *Dh*/+ heterozygotes, respectively. These results indicate that agenesis of the spleen, concomitant with the presence of the heterozygous nude gene, contribute to a high incidence of SMT in the low-SMT BALB/c mouse strain.

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