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Development and Characterization of the BALB/cNIV Mouse Strain[1]

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ABSTRACT

The strain BALB/cNIV/CrgI was developed by infecting BALB/c/CrgI mice with mouse mammary tumor virus from C3Hf mice. A BALB/c normal mammary duct was transplanted into the gland-free fat pad of a hormone-stimulated female C3Hf × BALB/c F_1 mouse. A hyperplastic alveolar nodule was found in the BALB/c ductal outgrowth and was transplanted into another hybrid gland-free fat pad. The resultant hyperplastic alveolar outgrowth was finally transplanted to female BALB/c mice. The hyperplastic alveolar outgrowth contained an exogenous, infectious mouse mammary tumor virus named the nodule-inducing virus, which was thought to be derived from the endogenous low oncogenic mouse mammary tumor virus found in C3Hf mice. The hyperplastic alveolar outgrowth-bearing BALB/c mice were inbred for four generations, and one family was selected as the strain BALB/cNIV/CrgI. It was found that (*a*) the mouse mammary tumor virus found in the BALB/cNIV strain was milk transmitted, but not transmitted by infected males; (*b*) the BALB/cNIV breeding females had a low tumor incidence (40%) and a longer latent period (14 months) than did female BALB/cfC3H mice (92% at 8 months); (*c*) the BALB/cNIV nodule outgrowths had low tumor-producing capabilities (50%) and longer latent periods (13.4 months) than did nodule outgrowths derived from female BALB/cfC3H mice (100% at 7.7 months).

INTRODUCTION

The low mammary tumor incidence C3Hf mouse strain carries a MuMTV[3] which is sometimes referred to as the NIV (13, 14, 23, 24, 27, 28). NIV was first found in the milk, HAN, and tumors of C3Hf mice (9, 15, 29). Because of its association with a low mammary tumor incidence and a high HAN incidence, NIV was presumed to induce nodules but not tumors (27, 28). It has also been classified as MuMTV-L (1–3, 18).

Since NIV has biological properties which are different from the high tumor-inducing, milk-transmitted MuMTV-S found in C3H mice, the virus was of great interest. Early attempts to study the infectivity of NIV were frustrated by the fact that NIV could not be readily transmitted to and maintained in BALB/c mice by either milk or mammary tumor extracts (7, 17, 29, 31). However, a complex tissue transplantation scheme was used which successfully established a low oncogenic exogenous MuMTV in BALB/c mice. This mouse strain became known as BALB/cNIV (14, 30, 38).

The BALB/cNIV strain has been a useful source of a low oncogenic MuMTV and has been used in a number of immunological and biochemical studies (5, 6, 8, 19, 20, 33, 34). However, in the 18 years since the development of the BALB/cNIV strain, we have published only brief accounts of the origin of these animals (14, 30, 38). Because of the continuing interest in BALB/cNIV mice, we provide here a more detailed description of the development and the biological characteristics of the BALB/cNIV strain.

MATERIALS AND METHODS

Mice. All mice were obtained from the breeding colony maintained by the Cancer Research Laboratory, University of California, Berkeley, CA.

Tissue Transplantation. Transplantation of mammary tissues into the cleared inguinal mammary fat pads was done by the technique described by DeOme *et al.* (10). All mice were cleared and transplanted at 3 weeks of age.

Virus Sources. Cell-free extracts of tumor and lactating mammary glands were prepared by homogenization in a Waring Blendor. The homogenized tissue was clarified (30 min at 2,200 × *g*), and the virus was pelleted (60 min at 11,800 × *g*). The pellet was resuspended in the calculated amount of 0.9% NaCl solution necessary for a concentration of 125-mg equivalents of wet weight tissue per ml.

Milk was obtained from 7- to 10-day lactating females by removing their litters overnight to allow milk storage. Prior to milking, 0.2 ml oxytocin (Pitocin, Parke-Davis Co., Seattle, WA; 10 units/ml) was inoculated i.p. The glandular areas were massaged with a cotton swab saturated with warm 0.9% NaCl solution prior to milking with a pulsating-suction device.

The milk was delipidated and clarified with TEN buffer (0.01 M Tris-HCl-0.001 M EDTA-0.1 M NaCl, pH 7.4), with the EDTA concentration increased to 0.025 M, by centrifugation for 10 min at 10,000 × *g*. Sucrose density gradient preparations of the supernatant were obtained by layering onto a 15 to 65% density interface, which in turn was layered onto a 15 to 65% linear sucrose gradient (3 hr at 130,000 × *g*). The virus band was collected by bottom puncture.

Blood was collected via tail bleeding, pooled, heparinized, and diluted with 0.9% NaCl solution prior to noduligenesis bioassay.

Noduligenesis Bioassay. All cell-free extracts and sucrose density gradient preparations were filtered through a 0.45-μm Millipore filter prior to noduligenesis bioassay. Either 25-mg equivalents of tumor and lactating mammary gland cell-free extracts, 0.01-ml equivalents of milk, or 0.1-ml equivalents of whole blood were inoculated i.p. into 3-week-old female or male BALB/c test mice.

All noduligenesis tests were done by the hormonal treatment of Nandi (22). Both male and female test mice were implanted s.c. on the dorsal side with hormone pellets containing 0.08 mg 17β-estradiol, 20 mg deoxycorticosterone acetate, and 5 mg cholesterol. Whenever male mice were used in the noduligenesis tests, they were gonadectomized before hormone pellet treatment. The first hormone pellet was removed after 6 weeks and replaced by another for the following 6 weeks. At the end of 12 weeks of hormonal stimulation, the second hormone pellet was removed and the mammary glands were allowed to regress for 5 weeks, at which time the animals were killed by cervical dislocation, the mammary glands were fixed in formalin for 24 hr, defatted in acetone, and stained as whole mounts with hematoxylin. After staining, the whole

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[3] The abbreviations used are: MuMTV, mouse mammary tumor virus; NIV, nodule-inducing virus; MuMTV-L, murine mammary tumor virus, low oncogenic; MuMTV-S, murine mammary tumor virus, standard; HAN, hyperplastic alveolar nodule; HPO, hyperplastic alveolar outgrowth.

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mounts were examined under the dissecting microscope for the presence of nodules, which is an indication that infectious MuMTV had been present in the original inoculum.

Cell Dissociation. Mammary glands from virgin and parous females were enzymatically dissociated, 10^5 cells were injected into gland-free fat pads, and the resultant outgrowths were classified as detailed by DeOme *et al.* (11).

RESULTS

Development of BALB/cNIV Mice. The BALB/cNIV strain was developed by using a complex program of mammary gland transplantation and breeding experiments which culminated in the selection of one subset of MuMTV-infected animals. Eighteen transplants of mammary ducts from 3-week-old female BALB/c mice were placed into gland-free mammary fat pads of hybrid female C3Hf × BALB/c F_1 mice. The hybrid females were allowed to litter twice and were then treated for 2 months with daily s.c. injections of 1 μg 17β-estradiol and 500 μg deoxycorticosterone acetate. At 18.5 months of age, the transplanted BALB/c mammary glands were examined for HANs. One of the 18 HAN transplants gave a HPO. The HPO was subdivided and transplanted into the gland-free fat pads of several groups of female BALB/c mice. One group of recipient female BALB/c mice was treated with 17β-estradiol and progesterone injected s.c. for 21 days, and then bred with male BALB/c mice. Three litters were developed into families by 4 generations of brother × sister matings. At this time one family was arbitrarily selected and maintained as the BALB/cNIV strain.

MuMTV Expression and Transmission in BALB/cNIV Mice. BALB/c mice do not normally express high levels of MuMTV or develop tumors or HANs (26, 32). The BALB/cNIV strain contains virus which can be detected in milk as whole virions containing MuMTV antigens and nucleic acids (5, 14, 32, 36). MuMTV antigens also can be detected in BALB/cNIV mammary glands, HPOs, and tumors using the immunoperoxidase technique (data not shown).

The pattern of MuMTV transmission in BALB/cNIV mice was studied using foster nursing and by backcrossing to BALB/c mice (Table 1). Milk transmission of MuMTV was demonstrated by the development of HAN in BALB/cfBALB/cNIV mice. The incidence of nodules in BALB/cNIV animals could be reduced by foster nursing the animals on BALB/c mice. In contrast to the C3Hf strain, the male BALB/cNIV mice were not capable of transmitting the NIV to their BALB/c × BALB/cNIV F_1 offspring.

Similar to the BALB/cfC3H strain (25), the BALB/cNIV blood could also transmit the agent (Table 2). As in the milk transmission, BALB/cNIVfBALB/c blood was not infectious.

These results provide biological evidence that the BALB/cNIV strain carries an exogenous, milk-transmitted virus which is not genetically transmitted as in the C3Hf strain. This hypothesis has been recently verified at the DNA level (32).

BALB/cNIV HPO Tumor Potential. The tumor potential of BALB/cNIV, BALB/cfBALB/cNIV, and BALB/cfC3H HANs were compared by transplanting individual HANs into gland-free mammary fat pads. HPOs from BALB/cNIV and BALB/cfBALB/cNIV mice had a lower tumor incidence and a longer mean age of tumor onset than did HPOs from BALB/cfC3H mice (Table 3).

Nodule-transformed Cells in BALB/cNIV Mammary Glands. An estimate of the frequency of nodule-transformed cells in BALB/cNIV and C3Hf mammary glands was obtained using the cell dissociation-transplantation technique (11, 12); 10^5 enzymatically dissociated cells from the mammary glands of nulliparous and multiparous females were injected into cleared fat pads. Cells from all 4 experimental groups grew out as HPOs (Table 4). Cells from multiparous animals more frequently resulted in HPOs than did cells from nulliparous animals. Cells from BALB/cNIV mice gave more HPOs than did cells from C3Hf mice at any age tested.

Table 1

Noduligenesis test of BALB/cNIV, BALB/cfBALB/cNIV, BALB/cNIVfBALB/c, and BALB/c × BALB/cNIV F_1 mice

Noduligenesis bioassay: the various strains were treated with hormones for 12 weeks, after which the hormones were removed and the mammary glands were allowed to regress for 5 weeks. The mice were killed, the mammary glands were fixed in formalin, defatted in acetone, stained with hematoxylin, stored in methyl salicylate, and read for HANs.

	Mice			Nodules	
Mouse	Generations	No.	No. with HANs	No.	No./ HAN-bearing mouse
BALB/cNIV	2, 3, 4, 5	66	59 (87)[a]	1134	19.2
BALB/cfBALB/cNIV	2, 3	29	22 (76)	226	10.3
BALB/cNIVfBALB/c	2, 3, 4, 5	40	0	0	0
BALB/c × BALB/cNIV F_1		17	0	0	0
BALB/cfC3H		4	4 (100)	76	19.0
C3Hf		61	0	0	0

[a] Numbers in parentheses, percentage.

Table 3

Tumor-producing capability of nodule outgrowths

HANs were selected from BALB/cNIV, BALB/cfBALB/cNIV, and BALB/cfC3H donors and were transplanted into the gland-free inguinal fat pads of BALB/c mice. Only tumors which developed in nodule outgrowths in the transplanted pads were included.

Donor HAN	No. of transplants	No. of tumors	% of tumors	Mean age (mo)
BALB/cNIV	68	34	50.0	13.4
BALB/cfBALB/cNIV	27	9	33.3	11.2
BALB/cfC3H	28	28	100.0	7.7

Table 2

Noduligenesis test in BALB/c mice inoculated with whole blood from BALB/cNIV and BALB/cNIVfBALB/c mice

Noduligenesis bioassay: 0.1-ml equivalents of heparinized blood from BALB/cNIV or BALB/cNIVfBALB/c mice were injected i.p. into 3-week-old BALB/c mice. The number of HANs per mice given injections was determined after 12 weeks of hormone treatment and 5 weeks of regression (see text).

Donor	HAN	Range	No. of mice with HANs/ total no. of mice	% of mice with HANs	No. of HANs/ HAN-bearing mouse	Tumor
Generation 3 BALB/cNIV	1060	0–111	36/43	83.7	29.4	0
Generation 3 BALB/cNIVfBALB/c	0	0	0/5	0	0	0

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BALB/cNIV Mammary HAN and Tumor Incidence. If, as intended, NIV had been transmitted from C3Hf to BALB/cNIV mice, the infected animals should have a high nodule incidence and a low tumor incidence relative to the MuMTV-S-infected BALB/cfC3H strain.

BALB/cNIV and BALB/cfBALB/cNIV mice have more HANs than either BALB/cfC3H or C3Hf animals, but the differences are not dramatic (Table 5). In contrast, breeding female BALB/cNIV mice have a lower tumor incidence (40%) than do BALB/cfC3H mice (92%) and a longer latency period. The C3Hf strain has the lowest tumor incidence (21%) and longest mean latency period (20 months).

MuMTV Transmission from Other Low-Incidence Strains. Repeated attempts to infect BALB/c mice with MuMTV from strains Af, RIIIf, and DBA/2eB, following the same strategy used to establish the BALB/cNIV strain were unsuccessful.

Table 4

Incidence of HPOs derived from injection of 10^5 dissociated mammary gland cells obtained from nulliparous and multiparous BALB/cNIV and C3Hf donors

Cell dissociation assays: mammary glands were removed from donor mice and were dissociated by means of appropriate enzymes. Aliquots containing 10^5 viable cells were injected into the gland-free inguinal fat pads of similar host mice. Outgrowths were classified as ductal or nodule outgrowths.

Substrains	No. of donors	Age of donors (mo)	No. of pads injected	No. of outgrowths		% of HPOs
				Ductal	HPO	
BALB/cNIV, nulliparous	22	1	76	71	4	5.3
	11	6	85	84	1	1.2
	6	12	46	25	21	46
BALB/cNIV, multiparous	8	4.5	78	57	21	26.9
	5	7	34	26	8	24
	6	9–11	50	18	32	64
C3Hf, nulliparous	29	1	74	74	0	0
	12	5–6	71	71	0	0
	12	12–15	104	98	6	5.8
C3Hf	6	5–6	32	32	0	0
	16	13–16	87	51	36	41.4

DISCUSSION

The MuMTV is a major factor in murine mammary tumorigenesis (1, 2, 4, 13, 16, 18, 21, 26). The classical milk-transmitted Bittner agent, or the exogenous MuMTV-S, is a high oncogenic virus (1). However, some mouse strains, such as C3H, RIII, A, and DBA/2, appear to carry a second MuMTV which has a lower oncogenicity (3, 15). The second virus, usually designated MuMTV-L, is generally uncovered when MuMTV-S is removed by foster nursing (13, 23, 24), and has been shown to be a genetically transmitted, endogenous virus in most strains. One form of MuMTV-L has been localized to chromosome 7 and is now designated MTV-1 (35, 37).

Because mammary tumorigenesis is effected by a number of interacting factors, the comparison of different MuMTVs is best accomplished by isolating the several variants on the same genetic background. This initially proved impossible in the case of the C3Hf NIV strain. However, the BALB/cNIV strain was established using the complex transplantation strategy described here.

During the first 8 generations, the tumor incidence in the BALB/cNIV strain was low (40% at 14 months) as compared to the BALB/cfC3H strain (92% at 8 months). After the first 8 generations, the tumor incidence in the BALB/cNIV colony at the Cancer Research Laboratory, University of California, Berkeley, slowly increased to as high as 80% after 16 months. The entire University of California, Berkeley BALB/cNIV colony was discarded in 1980. However, the BALB/cNIV colony which has been maintained in the Department of Pathology, University of California, Davis, School of Medicine, has had a tumor incidence of 24%, with a mean age of 15.7 months after 4 full generations.

The BALB/cNIV strain has the high incidence of HANs expected of a strain carrying a MuMTV-L. The average number of HAN animals were comparable to that observed in BALB/c mice infected with MuMTV-S (Table 1). This is also reflected in the rate at which dissociated mammary cells from BALB/cNIV mice form HPOs when injected into mammary gland-cleared fat pads. Since this is a test for latent nodule-transformed cells, we infer

Table 5

Incidence of HANs and mammary tumors in BALB/cNIV, BALB/cNIVfBALB/c, BALB/cfBALB/cNIV, BALB/cfC3H, and C3Hf multiparous and nulliparous mice

Mammary glands from the various mouse strains were fixed in formalin, defatted in acetone, stained with hematoxylin, and stored in methyl salicylate. The numbers of HANs and tumors were recorded.

Mouse type	Generations	No.	Age (mo)	No. with HANs	No. with tumors	Mean age of tumor-bearing mice (mo)	Nodules	
							Total no.	No./HAN-bearing mouse
BALB/cNIV								
Multiparous	1–8	80	10–23	76 (95)[a]	32 (40)	14	2459	32.4
Nulliparous	4–8	213	12–25	75 (35)	10 (5)	18	682	9.1
BALB/cNIVfBALB/c								
Multiparous	1–4	17	13–17	0	0		0	0
BALB/cfBALB/cNIV								
Multiparous	1–2	19	14–18	16 (84)	1 (5)	16	516	32.3
BALB/cfC3H								
Multiparous		49[b]			45 (92)	8		
Nulliparous		107			4 (4)	12	78	2.9
C3Hf								
Multiparous		29	14–24	19 (65)	6 (21)	20	358	18.8
Nulliparous		52	13–24	15 (29)	0		51	3.4

[a] Numbers in parentheses, percentage.
[b] BALB/cfC3H mammary tumor incidence in the breeding stock colony of the Cancer Research Laboratory, University of California, Berkeley, CA, January 1976.

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that BALB/cNIV mammary glands have as many nodule-transformed cells as do BALB/cfC3H mammary glands. However, it is quite clear, from the overall tumor incidence and from direct comparison of tumor rates of HAN outgrowths of BALB/cNIV and BALB/cfC3H mice, that hyperplastic cells from BALB/cNIV mice do not progress to cancer at the same rate.

Since the major variable between the high tumor incidence BALB/cfC3H strain and the low tumor incidence BALB/cNIV strain is the particular strain of MuMTV they transmit, these characteristics must be directly attributable to the virus. In view of the strategy used to develop the BALB/cNIV strain, it has been reasonable to assume that the virus in BALB/cNIV mice was directly transmitted from C3Hf mice. The exogenous virus found in BALB/cNIV mice does share antigenic and restriction endonuclease patterns with the C3Hf MuMTV (32, 36). However, significant antigenic and restriction site differences occur between the 2 viruses which suggest that the BALB/cNIV MuMTV is different from C3Hf NIV MuMTV (32, 36). Thus, the actual origin of the exogenous BALB/cNIV MuMTV is still in question. The BALB/cNIV virus could be the result of genetic drift during the last 18 years since the original transfer, or it could be the result of a recombinant between an endogenous BALB/c provirus and the exogenous NIV. Other alternatives must be considered.

Whatever the origin of the BALB/cNIV MuMTV, the importance of the BALB/cNIV mouse is reflected in the biological characteristics of the strain. The strain now carries and transmits a prototype MuMTV-L. Since it is in the BALB/c background, comparative studies with MuMTV-S can form the basis of an understanding of how these viruses lead to the neoplastic transformation.

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