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Viral Expression and Immunogenicity of CBA Mammary Carcinomas and their Hybrid Lines with an L-Cell Derivative (A9HT)*

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Abstract—*Somatic cell hybrid lines were obtained by Sendai virus mediated fusion of a highly tumorigenic variant of L-cell subline A9 of C3H mouse origin (A9HT) with two CBA mouse mammary carcinomas (SBfnHA and SBfnHC) that had been inoculated into CBAT6 mice. Hybridity was confirmed by the presence of biarmed marker chromosomes from the A9HT parent, and the total chromosome number, which in both hybrids (SBfnHA/A9HT and SBfnHC/A9HT) approximated the sum of the parental chromosomes. The absence of the T6 marker in both the hybrids confirmed that fusion was not between a stroma cell from CBAT6 mice and A9HT. Both hybrids showed spindle cell carcinoma morphology. Complement dependent cytotoxicity assays revealed that SBfnHA and A9HT expressed the MTV-associated antigens including one major structural component, gp52 and the MuLV structural components gp71 and p30 on the cell-surface, while SBfnHC expressed only gp71. The hybrid line SBfnHA/A9HT lost the surface expression of MTV gp52 and MuLVp30, and there was also decreased expression of gp71 in comparison with SBfnHA. In contrast, in SBfnHC/A9HT there was increased expression of gp71 over that of SBfnHC. All parental and hybrid lines had unchanged sensitivity to the cytotoxic effect of anti-H-2^k sera. Immunization of CBA mice with heavily irradiated either syngeneic SBfnHA or hybrid SBfnHA/A9HT cells, followed by challenge with viable SBfnHA cells revealed that SBfnHA was a highly immunogenic tumor, while the hybrid line SBfnHA/A9HT decreased in the immunogenicity of the parental SBfnHA line. On the other hand, immunization of CBA mice with heavily irradiated either syngeneic SBfnHC or hybrid SBfnHC/A9HT cells, followed by challenge with viable SBfnHC cells showed that SBfnHC/A9HT slightly increased the very low immunogenicity of the parental SBfnHC. These results are consistent with the possibility that MTV- and MuLV-associated cell-surface antigens influence the immunogenicity of CBA mammary carcinomas.*

INTRODUCTION

THE MAJORITY of mammary carcinomas produce a certain degree of transplantation resistance in syngeneic hosts, while a minority produce either no effect or a certain degree of enhancement of tumor growth [1]. The nature of the difference between the rejection versus enhancement inducing mammary carcinomas has not been elucidated.

We have previously demonstrated that superinfection with xenotropic endogenous mouse virus increases the immunogenicity of chemically induced tumors in rats [2]. Somatic cell hybrids between tumors and xenogenic [3] or allogenic [4–6] cells have also been used for increasing the immunogenicity of tumors. The immunologic rejection of hy-

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brid cells, resulting from the helper effects of foreign histocompatibility antigens, might initiate immunity against tumor-associated antigens of the tumor. However, such studies are often difficult to evaluate since they do not include the comparison of immunogenicity between inactivated hybrid cells and inactivated parental cells.

In the present studies we examined whether the immunogenicity of CBA mammary carcinomas could be increased by somatic cell fusion with H-2 identical allogenic cells, and the relationship of such immunogenicity to the presence of MTV (mammary tumor virus)- and MuLV (murine leukemia virus)-associated antigens.

MATERIALS AND METHODS

Animals

Two to three months old female CBA and (CBAT6T6 × C57BL)F_1 mice were derived from our own colony.

Cell lines

Cultured lines of CBA mouse mammary tumors, SBfnHA and SBfnHC have originated in female mice, foster-nursed on C3H (S means spontaneous. B stands for CBA, fn for foster nursing, H for the foster C3H mother, A and C for the serial name of the tumor). SBfnHA is an adenocarcinoma, and SBfnHC is a spindle cell carcinoma [7]. In order to distinguish between tumor and stroma cells, they were inoculated into CBAT6 F_1 mice homozygous for the T6 chromosome translocation marker, and the tumors that developed and designated SBfnHA(0T6) and SBfnHC(0T6) were re-explanted. The SBfnHA and SBfnHC are sensitive to 0.13–1 mM ouabain, and cannot grow in MEM containing 3 mM ouabain. A9HT (clone 3c) was developed by mutagenizing a highly tumorigenic variant of L cell subline A9 of C3H mouse origin with ethylmethanesulfonate. A9HT lacks the genetic information for the enzyme HGPRT-ase (hypoxanthine–guanine–phosphoribosyl-transferase), and therefore cannot grow in HAT medium. The mutagenized cells were selected stepwise for resistance to ouabain. The cloned and recloned line, designated cl 3 c is resistant to 6mM ouabain [8]. All lines were maintained in Dulbecco's modified MEM supplemented with 5–10% FCS and antibiotics.

Cell hybridization

Two hybrid lines were established by Sendai virus fusion of SBfnHA or SBfnHC derived from CBAT6 mice, and A9HT, as the following. Subconfluent monolayer cells of SBfnHA and SBfnHC in 75 cm^2 flasks (Falcon) were rinsed twice in cold RPMI, and 1000 HAU of β-propriolactone-inactivated Sendai virus (Stenman) in 0.5 ml was added to each flask, and the flasks were cooled to 4°C for 30 min. After washing twice, 5×10^6 trypsinized and twice washed A9HT cells were added in 1 ml of cold RPMI to the SBfnHA and SBfnHC preabsorbed by Sendai virus, and the mixtures were kept at 4°C for 30 min and then warmed to 37°C for an additional 60 min. Then Dulbecco's modified MEM supplemented with 5% FCS was added, and the cells were cultured at 37°C in a CO_2 incubator. The next day, the culture fluid was aspirated and replaced by HAT medium supplemented with 3 mM ouabain. The HAT medium was changed twice weekly. By the end of the second week of culture, well-isolated colonies of cells were detected, and two hybrid lines designated SBfnHA/A9HT and SBfnHC/A9HT were isolated.

Chromosome preparations

Metaphase spreads of cultured cells were prepared by the air-drying technique [9]. Colcemid (GIBCO, Grand Island N.Y.) was added to the culture in a final concentration of 0.04 μg/ml 2–3 hr before the cells were harvested. After hypotonic treatment (0.075 M KCl solution for 5 min), the pellet was fixed in acetic-methanol (1:3), spread on chilled wet slides, air-dried, and stained with alkaline Giemsa. Chromosomes of the *in vitro* lines were analyzed twice. The total number of chromosomes, and the proportion of bi-armed chromosomes and T6 marker chromosomes were estimated by direct counting under the microscope.

Light-microscopy and electron-microscopy (EM)

Tissues from each tumor arising after inoculation of cultured hybrid cells were fixed in 10% formalin, processed routinely and sections stained with hematoxylin–eosin (HE), Gomori's stain for reticulin and with Masson's trichrome stain for collagen. The low-speed centrifugation pellet of EDTA trypsin dispersed culture cells and tissues from tumors developing after inoculation of cultured cells were fixed with 2% glutaraldehyde in

Sorensen's buffer, and embedded in araldite (ARA) for EM. Sections were cut with an LKB ultratome II using freshly cut glass knives, double stained with uranyl acetate and lead citrate and viewed in Philips EM 301G.

Microcytotoxicity assays

Rabbit anti-MTV (Lot. 666) absorbed *in vivo* to eliminate all non-viral antibodies [10] was kindly given by Dr. J. Hilgers (Division of Genetics, The Netherlands Cancer Institute, Plesmanlaan, Amsterdam, The Netherlands). Rabbit anti-mouse MTV gp52 serum was kindly provided by Dr. D. P. Bolognesi (Dept. of Surgery, Duke University Medical Center, Durham, North Carolina). Rabbit anti-Friend gp71 serum was kindly given by Dr. W. Schäfer (Max-Planck-Institut für Virusforschung, Tübingen, Germany), and Goat anti-Rauscher virus p30 was obtained through the Office of Resources and Logistics, Virus Cancer Program, National Cancer Institute, Bethesda, Md. Mouse anti-H-2^k serum was obtained from ASW mice immunized with C3H spleen cells. Microcytotoxicity assay was performed as described previously [2]. Cytotoxic index (CI) exceeding 0.20 was regarded as positive. Three cultured lines: MBK (a methylcholanthrene-induced sarcoma in CBA mice), BALB/3T3 and JLS-V9 were used as control target cells.

Immunization with irradiated cells

One million parental or hybrid cells suspended in 2 ml medium were layered in a plastic petri dish (35 x 10 mm, Falcon). They were irradiated with 10,000 rad using a Stabilipan, Siemens X-ray source, under the following conditions: 15 mA, 220 kV, 1 mm Al filter, at 22 cm focus distance and 960 rad/in dose rate. The dose was calibrated by an attached Phillips dosimeter. For immunization, the irradiated 10^6 cells were inoculated subcutaneously into CBA mice, once a week for 3 weeks. Seven days after the last immunization, all immunized and untreated mice received 400 rad whole-body irradiation, and the following day were subcutaneously challenged with between 5×10^2 and 10^5 viable tumor cells. Developing tumors were followed by regular caliper measurements. They were followed for a period of 6 months.

RESULTS

Identification of hybrid cell lines

The chromosomal constitution of the parental and hybrid cells is shown in Table 1. The modal chromosome number of SBfnHA was 73, while that of SBfnHC was 85, however the latter had a wide range of chromosome numbers 40–162. A9HT had between 48 and 57 total chromosomes with a mode of 22 biarmed chromosomes. SBfnHA/A9HT and SBfnHC/A9HT hybrids were identified by the total chromosome number and the presence of biarmed chromosomes from the A9HT parental cells. SBfnHA/A9HT and SBfnHC/A9HT had a wide range of chromosome number which approximated the sum of the parental chromosomes, although the modal numbers were below that expected for the sum of the two parental cells. SBfnHA/A9HT retained the biarmed chromosomes of A9HT (mode 26), while SBfnHC/A9HT had a decreased number 16 of biarmed chromosomes. Neither of the hybrid lines contained any T6 marker chromosomes, thus eliminating the possibility that the hybrids were derived from fusion of

Table 1. *Chromosome constitution of parental and hybrid cells*

	Total chromosome number		No. of biarmed chromosomes	
Cell line*	Range	Mode	Range	Mode
Parents				
SBfnHA	70–77	73	—	—
SBfnHC	40–162	85	—	—
A9HT	48–57	53	18–24	22
Hybrids				
SBfnHA/A9HT	69–158	112	20–30	26
SBfnHC/A9HT	107–138	123	11–21	16

*Twenty metaphases were examined for each cell line.

A9HT cells with contaminating stromal host cells.

Morphological character of hybrid lines

SBfnHA/A9HT had an epithelial morphology, and SBfnHC/A9HT consisted of cells intermediate between epithelial and fibroblastic morphology. However EM findings revealed that both of the hybrid cells had the morphological character of epithelial cells with abundant tonofilaments. To test the tumorigenicity of both hybrids, they were inoculated into 400 rad irradiated CBA newborn mice. The tumor take incidence increased gradually after serial passage *in vivo*, and eventually tumors grew in non-irradiated young adult CBA mice. Histological examination of both hybrid tumors showed pleomorphic spindle cell tumors with heavy pericellular reticulin and collagen (Figs. 1 and 2). On the other hand, EM examination showed that the cells still maintained an epithelial morphology characterized by plentiful tonofilaments, occasional secretory droplets, tight junctions and mini acinus formation (Figs. 3 and 4), and they were diagnosed as spindle cell carcinomas like the parental SBfnHC.

Antigen expression of parental and hybrid lines

To assess the expression of MTV- and MuLV-associated antigens and the major histocompatibility antigen. H-2^k on the cell-surface of parental and hybrid lines, complement cytotoxicity assays were performed using antisera against MTV and its major structural component gp52 and antisera against MuLV structural components, gp71 and p30 and anti H-2^k serum (Table 2). One of the CBA mammary tumor SBfnHA showed sensitivity to anti-whole MTV, anti-MTV gp52 and anti-MuLV gp71, p30 sera, while the other mammary tumor SBfnHC reacted only to anti-gp71 serum. The C3H L-cell derived A9HT was highly sensitive to anti-gp71 serum, and also reacted with anti-MTV, gp52 and anti-p30 sera. MBK, BALB/3T3 and JLS-V9 cells used as control target cells did not show any detectable sensitivity to all antisera except that MBK was weakly sensitive to anti-gp71 serum. One of the hybrid lines SBfnHA/A9HT showed sensitivity to anti-gp71 serum, but had none of the reactivity of both its parents to anti-MTV, gp52 and anti-p30 sera. On the other hand, SBfnHC/A9HT showed higher sensitivity to anti-gp71 serum than the parental SBfnHC, but like the latter there was no reactivity to other antisera. All cell lines showed almost equal sensitivity to anti H-2^k serum. These results demonstrate that SBfnHA expresses both MTV- and MuLV-associated cell-surface antigens, while SBfnHC does not express these antigens except MuLV gp71. Furthermore, the SBfnHA/A9HT hybrid has lost the SBfnHA parental expression of MTV- and

Table 2. Cytotoxic sensitivity of parental and hybrid cell lines to anti-MTV, gp52, anti-MuLV (gp-71, p30) and anti-H-2^k sera

Cell line	Cytotoxic titer* with: Anti-MTV	Anti-MTV gp52	Anti-MuLV gp71	Anti-MuLV p30	Anti-H-2^k†
Parents					
SBfnHA	80	40	2560	20	1280
SBfnHC	‡	—	320	—	1280
A9HT	160	80	10240	2560	2560
Hybrids					
SBfnHA/A9HT	—		1280	—	1280
SBfnHC/A9HT		—	5120	—	1280
Controls					
MBK§	—		20	—	N.D.‖
BALB/3T3	—	—	—	—	N.D.
JLS-V9		—	—		N.D.

*Reciprocal of serum dilution producing more than 0.20 of cytotoxic index.
†ASW anti-C3H serum was used.
‡No positive reaction at 1:10 serum dilution.
§A methylcholanthrene-induced CBA sarcoma.
‖Not done.

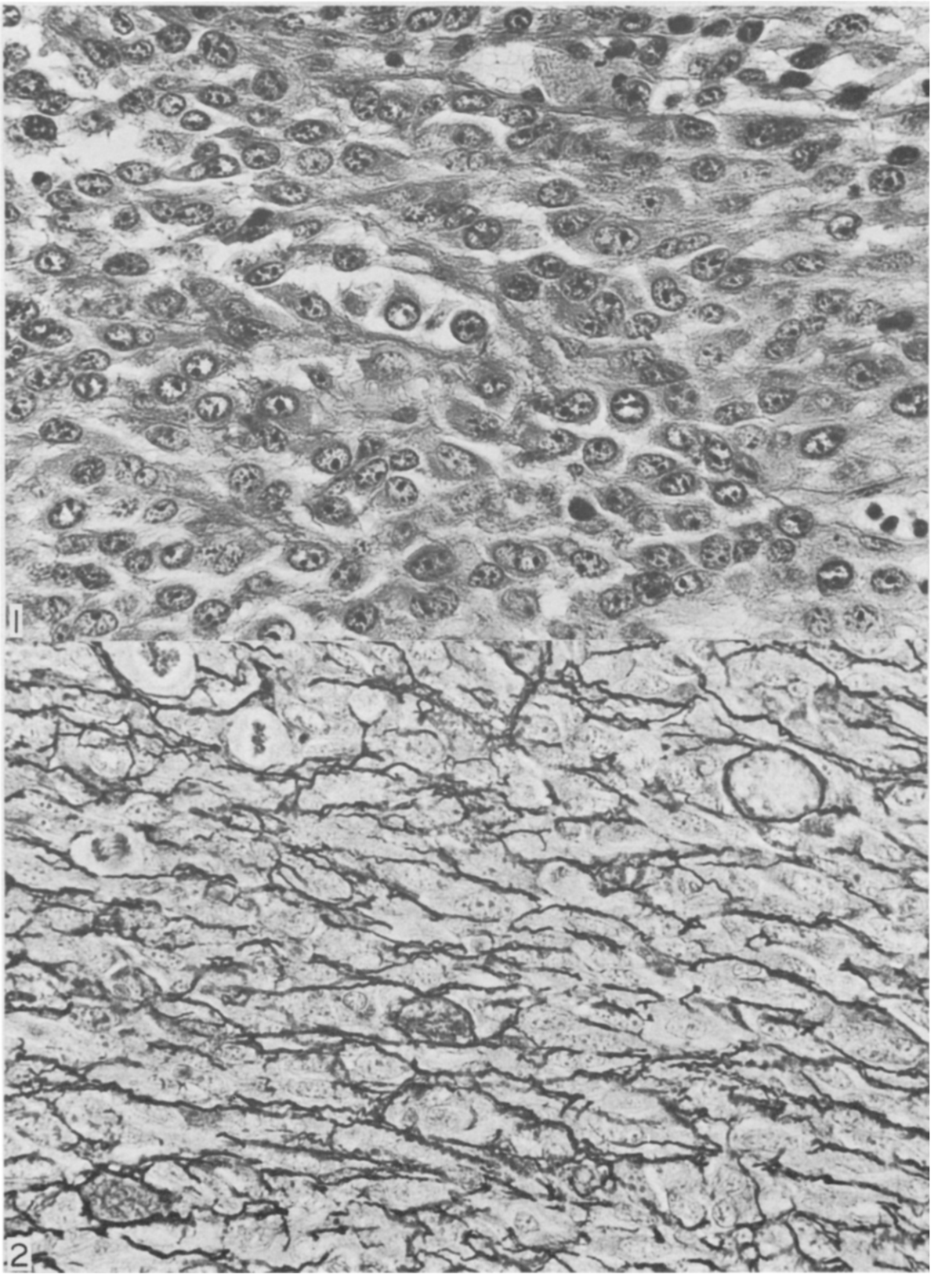

Fig. 1. SBfnHA/A9HT (Hematoxylin and Eosin × 500). This shows a moderately pleomorphic predominantly spindle celled tumor in which there is moderate nuclear anisocytosis.

Fig. 2. SBfnHC/A9HT (Gomori stain for reticulin × 500). This shows a heavy pattern of reticulin present in many areas around individual cells. The pattern of collagen fibers was similar.

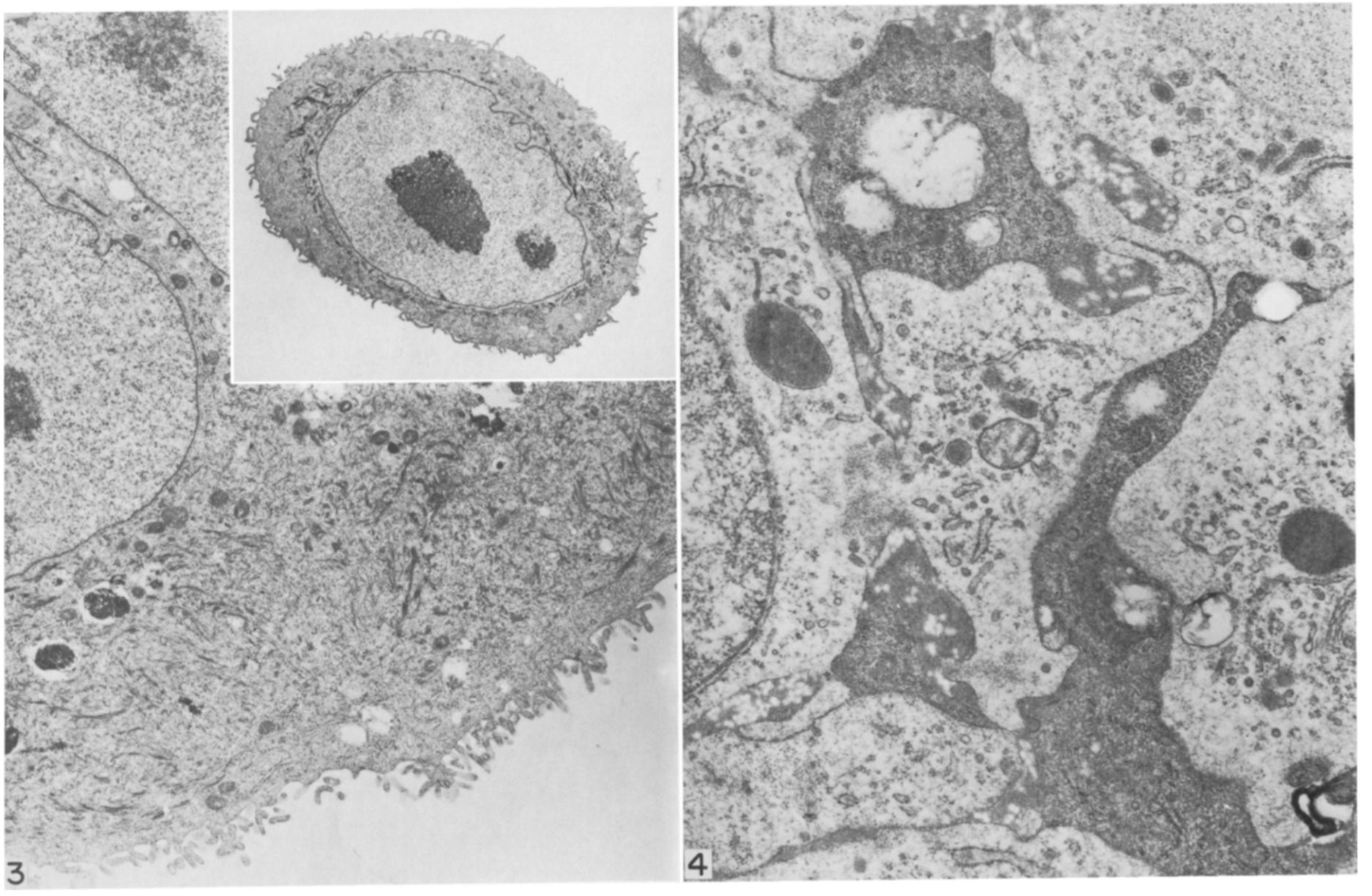

Fig. 3. Electron micrograph showing cytoplasm of SBfnHA 19HT (×16,870). Note large numbers of tonofilaments present in the cytoplasm. The nucleus is active with abundant euchromatin and portions of three nucleoli are present. The insert shows LP view of single cell (×5400). Note active cell border with abundant short microvilli. The nucleolus is prominent and much fibrillar material is present in the cytoplasm.

Fig. 4. Electron micrograph showing cytoplasm of SBfnHC 19HT (×29,400). Note presence of membrane bound secretory droplets. A few cisternae of rough endoplasmic reticulum are present and Golgi apparatus is seen.

MuLV-antigens except for gp71, and expression of gp 71 antigen on SBfnHC/A9HT is greater than that of the parental SBfnHC.

Immunogenicity of parental and hybrid lines

Immunogenicity of parental and hybrid lines was examined by tumor rejection tests against each CBA parental mammary carcinoma line. Female CBA mice were immunized with irradiated cells three times at weekly intervals. Seven days after the last immunization, the mice received 400 rad wholebody irradiation and, on the following day, they were challenged with different numbers of viable parental CBA mammary carcinoma cells (Table 3). CBA mice immunized with irradiated A9HT and non-immunized CBA mice failed to reject 10^4 and 10^5 SBfnHA cells. Mice immunized with irradiated SBfnHA cells completely rejected 10^4 and 10^5 viable SBfnHA cells, while mice immunized with irradiated SBfnHA/A9HT rejected 10^4 viable SBfnHA cells, but 5 out of 8 mice could not reject 10^5 viable tumor cells. In contrast, none of the mice immunized with irradiated SBfnHC, SBfnHC/A9HT or A9HT cells showed any significant rejection reaction against viable SBfnHC cells. However, there was a significant prolongation of survival time in mice immunized with SBfnHC/A9HT. These results demonstrate that SBfnHA is a highly antigenic tumor, while SBfnHC is a

Table 3. Comparison of immunogenicity between inactivated parental and hybrid cell lines in CBA mice

Immunization*			Challenge†		
Cell line	Dose	Times	Cell line	Dose	Lethal growth (MSD)‡
SBfnHA	10^6	3	SBfnHA	10^4	0/2
SBfnHA	10^6	3	SBfnHA	10^5	0/4
					0/6
SBfnHA/A9HT	10^6	3	SBfnHA	10^4	0/4
SBfnHA/A9HT	10^6	3	SBfnHA	10^5	5/8 (72.0)
					5/12
A9HT	10^6	3	SBfnHA	10^4	2/2 (52.5)
A9HT	10^6	3	SBfnHA	10^5	2/2 (45.0)
					4/4
None			SBfnHA	10^4	2/2 (54.0)
None			SBfnHA	10^5	3/3 (42.0)
					5/5
SBfnHC	10^6	3	SBfnHC	5×10^2	3/4 (101.0)
SBfnHC	10^6	3	SBfnHC	5×10^3	8/8 (58.6)
					11/12
SBfnHC/A9HT	10^6	3	SBfnHC	5×10^2	6/9 (135.3)
SBfnHC/A9HT	10^6	3	SBfnHC	5×10^3	7/8 (71.5)
					13/17
A9HT	10^6	3	SBfnHC	5×10^2	3/4 (101.7)
A9HT	10^6	3	SBfnHC	5×10^3	4/4 (47.0)
					7/8
None			SBfnHC	5×10^2	4/4 (99.0)
None			SBfnHC	5×10^3	4/4 (57.0)
					8/8

*Heavily irradiated (10,000 rad) cells were inoculated into CBA mice.

†Challenge 8 days after final immunization. Hosts were irradiated 400 rad 24 hr before tumor inoculation.

‡Mean survival time in days.

low antigenic tumor. In addition, the immunogenicity of the SBfnHA/A9HT hybrid is decreased in comparison with its parental SBfnHA, whereas the SBfnHC/A9HT hybrid shows an increased immunogenicity in comparison with its parental SBfnHC cells.

DISCUSSION

MTV antigens on the surface of mouse mammary epithelial cells as well as in various lymphoid tissues have been detected by immunofluorescence and immunoelectron microscopy [10, 11]. Recently, specific antiserum against the MTV polypeptide gp52 has become available, and gp52 was demonstrated on the surface of the cells from mice with high mammary tumor incidence [12]. We have observed gp52 in mammary tumors in mice with low incidence. In addition to MTV antigens, mouse mammary tumors express MuLV-associated antigens [10], gp71 [12], and in some cases we have observed p30 antigen.

Our present experiments show that a mammary tumor, SBfnHA, derived from a CBA mouse foster-nursed by a C3H mother, expressed MTV-associated antigens gp52 and MuLV-associated antigens gp71 and p30 antigens, while another such tumor, SBfnHC, expressed only gp71 antigen. Although it has long been accepted that mice with a high incidence of mammary tumors may be tolerant to the virus and unable to respond immunologically to MTV, recent reports show that mice with a low tumor incidence and even MTV-positive mice do respond immunologically to both the intact MTV and to MTV antigens on the membranes of mammary tumor cells [13]. Similar kinds of natural immunity were also induced to the major virion envelope glycoprotein gp71 of MuLV [14]. Our present data show that a mammary tumor in CBA, SBfnHA expressing MTV-gp52 and MuLV-gp71 and p30 is immunogenic to syngeneic hosts, while immunization with another mammary tumor, SBfnHC which does not express MTV-antigens, and MuLV-p30 antigen, does not induce any protection against the identical tumor challenge in syngeneic mice. These findings suggest that MTV- and/or MuLV-associated antigens may influence the immunogenicity of mouse mammary tumors. To confirm this possibility, hybrid cells between SBfnHA or SBfnHC and an allogeneic line, with the same major histocompatibility antigen (A9HT) were prepared in this study. The hybrid line, SBfnHA/A9HT lost expression of MTV-associated antigens including gp52 and MuLV-p30 antigen, and showed decreased immunogenicity compared to the parental SBfnHA cells. In contrast, the other hybrid line, SBfnHC/A9HT expressed more gp71 antigen than the parental SBfnHC cells, and the mice immunized with inactivated SBfnHC/A9HT cells demonstrated increased survival time when compared to mice immunized with inactivated SBfnHC cells. All these data suggest that MTV- and/or MuLV-associated antigens may play an important role in inducing immunogenicity of mouse mammary tumors. In the case of SBfnHC/A9HT, one cannot rule out the possibility that minor histocompatibility antigen differences between A9HT cells and SBfnHC cells play a role in prolonging the survival of mice challenged with viable SBfnHC. However, this possibility seems unlikely in view of the fact that hybrid cells (SBfnHC/A9HT) grew well in CBA mice, and could not induce immunity at all.

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