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Serological Definition of Individually Distinct Surface Antigens in Mouse Mammary Carcinomas*

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Abstract—*For serological analysis of surface antigens in mouse mammary carcinoma, 13 cultured lines were established from spontaneous and MTV-induced tumors of 6 different mouse strains. Six of the cultured lines, SHK of C3H, Ta3Ha and TA3St of A, S2Y of ABY, SBfnHB and SBfnHD of C3H foster-nursed CBA origin were highly sensitive to polyvalent anti-MTV and anti-MTVgp52 sera, and this was invariably correlated with the presence of MTVgp52 and* p27 *antigens in the cell extracts. All cultured lines were sensitive to anti-MuLVgp70 serum, and several lines also contained considerable amounts of MuLVp30 antigen in the cell extracts.*

By immunization with cultured or in vivo *passaged tumor cells, 5 of 10 lines tested, SHF of C3H, S3W of ASW, S2Y of ABY; and SBfnHA and SBfnHB of C3H foster-nursed CBA origin could raise antisera containing complement-dependent cytotoxic antibodies to them in syngeneic or semisyngeneic mice. All the antisera contained antibodies against multiple specificities, although they were not cross-reactive to normal cells of C3H and BALB/c fetal origin, and normal spleen cells of C3H, DBA/2 and C57BL origin. One specificity of anti-S3W, anti-S2Y, anti-SBfnHA and anti-SBfnHB sera is cross-reactive for several leukemias and sarcomas, and the second specificity of anti-S3W, anti-SBfnHA and anti-SBfnHB sera is cross-reactive for polyoma virus-induced tumors. These antisera, after intensive absorption with leukemias and polyoma virus-induced tumor cells, and anti-SHF serum, demonstrated the third specificity which is cross-reactive for other mammary carcinomas and a C3H sarcoma. Further absorption with mammary carcinomas showed that anti-S3W and anti-SBfnHA sera have a fourth specificity, which had an absolute restriction to the immunizing tumor cells. These results suggest that individually distinct surface antigens were detectable in mouse mammary carcinomas by serological assays after absorption with cross-reactive cell lines.*

INTRODUCTION

THERE is increasing evidence that tumor cells may contain several different types of antigens. Mouse mammary carcinomas also have a wide complexity of antigens. The appearance of mammary carcinomas in most mouse strains is dependent upon infection by the mouse mammary tumor virus (MTV), and they express antigens related to the synthesis and release of viral particles [1–4]. Mouse mammary carcinomas also express 'normal' antigens, such as a differentiation antigen called MME [5], Thy 1.2 antigen [6, 7], alloantigen MM [8] and fetal antigens [9, 10]. Further, they may contain endogenous murine leukemia virus (MuLV)-associated antigens [2–4].

In addition to these common antigens, some mammary carcinomas express individually distinct tumor-specific surface antigens unrelated to MTV. They have been detected by means of transplantation techniques [11–13], colony inhibition assay [14], lymphocytotoxicity assay [7] and antibody-dependent cell-mediated cytotoxicity assay [15].

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The basis for this unique specificity has not been explained, nor have the target antigens involved been defined serologically or chemically. To initiate a serological analysis of the unique surface antigens of mouse mammary carcinomas, we have established several carcinoma cell lines in tissue culture [16]. Using these cell lines and syngeneic antisera, we have characterized cell surface antigens of mouse mammary carcinomas by the microcytotoxicity assay.

MATERIALS AND METHODS

Animals

Syngeneic mice of C3H, A, ACA, ASW, ABY, CBA, AKR, C57BL and DBA/2 are derived from the colony of Tumor Biology Department in Karolinska Institutet. F_1 hybrid mice used include C3H × ASW, A × C3H, ASW × C57BL, A × CBA and C57BL × ABY.

Cell lines

A list of cell lines used in our experiments is presented in Table 1. The morphologic details of the cultured mammary tumor lines and the derived tumors will be described elsewhere [16]. In these studies, mammary carcinoma lines, less than 50 generations *in vitro*, were used. All cultured cells were maintained *in vitro* in MEM (Eagle's minimum essential medium) with Earle's salts, supplemented with 5–10% fetal calf serum (Gibco) and antibiotics. Normal spleen cells used were derived from the C3H, DBA/2 and C57BL strains of mice.

Antisera to MTV- and MuLV-associated antigens

Rabbit anti-MTV (Lot 666) absorbed *in vivo* [2], and rabbit anti-MTVgp52 serum (Lot 12875) (kindly provided by Dr. D. P. Bolognesi, Dept. of Surgery, Duke University Medical Center, Durham, North Carolina, U.S.A.) were used. Rabbit anti-Friend gp70 serum was kindly provided 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, U.S.A.

Antisera to mammary carcinomas

Both viable cultured cells and irradiated *in vivo* passaged tumor cells were used for antiserum production. Syngeneic or semisyngeneic mice of either sex were immunized by injection of 10^4 viable cultured tumor cells in one hind foot pad. SHF, S3W, SBfnHA and SBfnHB did not produce tumors at this dose. On the other hand, SHK, S6C, S40C, S2Y, SBfnHC and SBfnHD grew within 1 month. These tumors were then resected by amputation. Injections were continued by 6 weekly s.c. challenges of increasing numbers of viable cultured cells, 10^4–10^6. Alternatively, syngeneic or semisyngeneic mice were immunized by injection of more than 10^6 irradiated (10,000 rad), *in vivo* passaged tumor cells, once weekly for 10–20 weeks. Mice that remained tumor-free through the immunization period were bled individually 1 week after the final immunization for the retro-orbital sinus, and their serum titered on the immunizing tumor cells. Similar titered antisera to each tumor were pooled and used for analysis of surface antigens.

Preparation of cell extracts

The cells of monolayer cultures were treated by Trypsin–EDTA solution (Gibco, Glasgow, Scotland), and washed 3 times with phosphate-buffered saline. Twenty per cent (v/v) suspensions were sonically treated with a Rapidis Ultrasonic Disintegrator A350G (Ultrasonics Ltd. Lamda Kemila, Stockholm) equipped with a microtip in an ice-water bath for a total of 60 sec. Sonically treated extracts were incubated at 37°C for 15 min with Triton X 100 at a final concentration of 0.1%, clarified by centrifugation at 3000 rev/min for 45 min at 4°C, and frozen at −20°C until assayed.

Purification of viral proteins

Dr. R. Nusse (Division of Virology, The Netherlands Cancer Institute) kindly provided the purified MTVgp52 and MTVp27 proteins from virions of the Mm5mt/cl cells. These purified virions were provided under contract with the Viral Cancer Program of the National Cancer Institute (Dr. J. Gruber). The purification method was described in [17]. MuLVp30 was a gift of Dr. M. Strand (Johns Hopkins University, Baltimore, U.S.A.).

Protein iodination

Iodination of MuLVp30, MTVp27 and MTVgp52 was performed by a slightly modified method first described for RNA tumor virus proteins of the C type by Strand and August [18]. The reaction was carried out at room temperature. It was started by adding 25 µl of 0.5 M $NaHPO_4$ (pH 7.5), 1 mCi Na ^{125}I and 10 µl chloramine-T (2 mg/ml in

Table 1. *Cell lines used in the experiments*

Cell lines	Strain	Oncogenic agents	Histology	Mode of propogation
SHF	C3H	Spontaneous (MTV)	Mammary adenocarcinoma	Culture
SHK	C3H	Spontaneous (MTV)	Mammary adenocarcinoma	Culture
SHN	C3H	Spontaneous (MTV)	Mammary carcinoma	Culture
Ta3Ha	A	Spontaneous (MTV)	Mammary carcinoma	Culture
TA3St	A	Spontaneous (MTV)	Mammary carcinoma	Culture
S6C	ACA	Spontaneous (MTV)	Mammary adenocarcinoma	Culture
S40C	ACA	Spontaneous (MTV)	Mammary carcinoma	Culture
S3W	ASW	Spontaneous (MTV)	Mammary adenocarcinoma	Culture
S2Y	ABY	Spontaneous (MTV)	Mammary adenocarcinoma	Culture
SBfnHA	CBA	C3H foster nurse	Mammary adenocarcinoma	Culture
SBfnHB	CBA	C3H foster nurse	Mammary adenocarcinoma	Culture
SBfnHC	CBA	C3H foster nurse	Mammary carcinoma	Culture
SBfnHD	CBA	C3H foster nurse	Mammary carcinoma	Culture
BW5147	AKR	Gross leukemia v.	Lymphoma	Ascites and Culture
YWA	ASW	Moloney 1. virus	Lymphoma	Ascites and Culture
YAC	A	Moloney 1. virus	Lymphoma	Ascites and Culture
YBA	CBA	Moloney 1. virus	Lymphoma	Ascites and Culture
RBL-5	C57BL	Rauscher 1. virus	Lymphoma	Ascites and Culture
ALC	C57BL	Radiation 1. v.	Lymphoma	Ascites and Culture
EL-4	C57BL	Benzpyrene	Lymphoma	Ascites and Culture
L1210	DBA/2	Methylchoranthrene	Lymphoma	Ascites and Culture
P815	DBA/2	Methylchoranthrene	Mastocytoma	Ascites and Culture
SEWE	ASW	Polyoma virus	Sarcoma	Ascites and Culture
SESO	A	Polyoma virus	Sarcoma	Ascites and Culture
SEAB	A	Polyoma virus	Sarcoma	Culture
SEYFa	ABY	Polyoma virus	Sarcoma	Ascites and Culture
Ha2	CBA	Moloney sarcoma v.	Sarcoma	Culture
MSB	C57BL	Moloney sarcoma v.	Sarcoma	Culture
MSWBS	ASW	Methylcholanthrene	Sarcoma	Ascites and Culture
MDAY	(A × DBA) F_1	Methylcholanthrene	Sarcoma	Ascites and Culture
MBK	CBA	Methylcholanthrene	Sarcoma	Culture
MBL	CBA	Methylcholanthrene	Sarcoma	Culture
MC57M	C57BL	Methylcholanthrene	Sarcoma	Culture
A9HT	C3H	Spontaneous	Sarcoma	Culture
JLS-V9	BALB/c	Fetal origin	Bone marrow derived	Culture
BALB/3T3	BALB/c	Fetal origin	Fibroblasts	Culture
C3HEF1	C3H	Fetal origin	Fibroblasts	Culture

0.1 M $NaHPO_4$, pH 7.5) to 15 μl viral protein (0.4–0.6 mg/ml) solution. After incubating for 1 min. 5 μl $Na_2S_2O_5$ (20 mg/ml in 0.1 M $NaHPO_4$, pH 7.5) and 50 μl 0.1 M KI were added to stop the reaction. The reaction mixture (except 5 μl, taken out for calculation of specific activity) was then applied to a Sephadex G-25 fine column (Pharmacia, Uppsala, Sweden) of about 10 ml, washed with at least 15 ml TEN buffer (20 mM Tris–HCl, pH 7.6, 1 mM EDTA, 100 mM NaCl). The column was presaturated with 1 ml of 20 mg/ml and equilibrated with at least 20 ml of 1 mg/ml crystalline bovine serum albumin (BSA). Elution was performed with 1 mg/ml crystalline BSA in TEN buffer and fractions of 0.5 ml were collected. The fraction or the two fractions with the highest radioactivity (usually fractions 4–6) were used. The viral proteins were continuously stored on ice in a cold room.

Calculation of specific activity of labeled viral proteins

Calculation of specific activity of labeled protein was performed by a TCA precipitation assay. Five μl of the reaction mixture was diluted in 1 ml of 1 mg/ml crystalline BSA in TEN buffer. Ten μl for this solution was counted (*in duplo*). Another 10 μl (also *in duplo*) was added to 50 μl of 2 mg/ml BSA (carrier protein) in TEN buffer. One ml of a 10% TCA solution was added. After a 45-min incubation on ice, the mixture was filtered on glass-microfibre filter (GF/C, Whatman) and the pellet was washed with 5 ml of cold 5% TCA. The filter with the precipated protein was counted. From the total counts (first tube) and the precipitated counts (filter) the percentage of binding of ^{125}I and the specific activity of the labeled protein were calculated. From the pooled peak fractions, 5 μl was taken and diluted in 1 ml of a 1 mg/ml crystalline BSA solution in TEN buffer. This diluted protein was treated in the same way by the TCA method. The recovery of the labeled protein after separation on the column was calculated from the precipitated counts. The labeled protein was never stored undiluted, but always diluted to 100 ng/ml with mg/ml of a crystalline BSA in TEN buffer. Before use in the RIA, the protein was diluted 4× with a dilution of normal rabbit serum in 2 mg/ml crystalline BSA in TEN buffer (1–9).

Radioimmunoassay

Serial dilutions of test samples (1:2 or 1:3 steps) were made in polystyrene tubes in a volume of 150 μl. The 2 mg/ml crystalline BSA in TEN buffer contained 0.2% Triton X-100 to solubilize the viral proteins. Ten μl rabbit anti-virus serum diluted towards the 50% binding level, was added to the dilutions of the test samples, followed by incubation at 37°C for 1 hr. Subsequently, 40 μl (1 ng) ^{125}I-labeled viral protein with a specific activity of $2–8 \times 10^4$ counts/min/ng, was added and the reaction mixture was again incubated for 1 hr at 37°C. An excess of goat anti rabbit IgG was added (30 μl undiluted serum from Meloy Laboratories) and incubation was carried out first for 1 hr and second for at least 12 hr at 4°C. A half ml of cold TEN buffer was used for washing and the pellets spun down at 3000 rev/min for 20 min in an MSE centrifuge. Washing was repeated twice. The pellet was counted in a Packard or Philips Auto Gamma Analyzer. In each test a standard of purified protein dilutions (ranging from 0.1–100 ng) was utilized.

Protein determination

Tubes were filled with 500 μl distilled water and 10 μl of the sample. A solution of 2% (w/v) Na_2CO_3, 0.5% SDS in 0.1 N NaOH was freshly added to a solution of 0.5% (w/v) $CuSO_4.5H_2O$ in 1% (w/v) sodium citrate in a ratio of 50:1 and 2 ml of this mixture was added to each tube and shaken immediately. A 10-min incubation followed. Immediately after addition of 200 μl phenol reagent freshly diluted in distilled water (1:1), the media were shaken and incubated for 30 min at 37°C. The samples were read on a spectrophotometer at 750 nm. (For details, see Lowry *et al.* [19].) A standard range of 0–100 μg crystalline BSA was utilized.

Microcytotoxicity assays and absorption tests

The microcytotoxicity assay was performed as follows: 2 μl serial antiserum dilutions from 1:10 in BSS (balanced salt solution) + 1% gelatin (BSS/gel) were injected under paraffin oil into rings of microtrays (Møller-Coats A/S, Moss, Norway) with an automatic Hamilton Syringe. One thousand target cells in 1 μl were added to each droplet and the plates were incubated for 20 min at 37°C. Subsequently 1 μl rabbit complement was added at a dilution of 1:3. Plates were further incubated for 45 min. thereafter 0.5 μl trypan blue was added to each drop and the results

were read microscopically. Cytotoxic index (*CI*) was calculated as follows: $CI = 1 - t/c$ (t: the percentage of unstained cells in the test sample, c: the percentage of unstained cells in the control sample). *CI* exceeding 0.2 was regarded as positive. All tests have been done at least twice, and the titers were within two dilution difference.

In order to remove all antibody activity against a certain cell type, serial absorption was carried out as follows: 1 vol of undiluted antiserum was incubated with 1 or 2 vols of cell pellet for 30 min at 37°C and 30 min at 4°C with occasional stirring. The absorbing cells were discarded after centrifugation. This procedure was repeated twice until the complete elimination of all detectable cytotoxic activity against the absorbing cell.

RESULTS

Expression of MTV- and MuLV-associated antigens by cultured mammary carcinomas

The cultured cell lines of mouse mammary carcinomas were assayed for MTV and MuLV expression by (a) microcytotoxicity assays with polyvalent anti-MTV serum, antiserum to MTV major envelope antigen gp52 and with antisera to the major structural MuLV antigens gp70 and p30, and (b) radioimmunoassays for MTVgp52 (major envelope protein), MTVp27 (major core protein) and MuLVp30 in cell extracts (Table 2). All mammary carcinomas except the SBfnHC were sensitive to anti-MTV and/or MTVgp52 sera with cytotoxic titers ranging

Table 2. Expression of MTV- and MuLV-associated antigens by mammary carcinoma lines

Cell lines	Direct cytotoxicity assays*			Radioimmunoassays†		
				Antigen concentration in cell extracts		
	Anti-whole MTV	Anti-MTV gp52	Anti-MuLV gp70	MTV gp52	MTV p27	MuLV p30
SHF	160	20	1280	<5.5	<9.1	1458
SHK	20,480	5120	640	1092	1555	312
SHN		160	160			
TA3Ha	320	160	2560	201	194	774
TA3St	604	1280	5120	389	2055	909
S6C	10	80	640	21	<12.1	1.4
S40C	80	160	2560	<1.7	<7.5	349
S3W	10	10	1280	<1.6	8	442
S2y	20,480	1280	640	2195	2019	1.0
SBfnHA	80	20	2560	<3	<20	313
SBfnHB	10,240	40	2560	125	63	295
SBfnHC	—‡	—	320			
SBfnHD	320	2560	640	>1587	>2125	4
BW5147	—		20,480			
RBL-5	—		20,480			
L1210	320	40	2560			
SEWE	—	—	640			
SEAB		—	2560		<8.0	<2.5
MSB	—	—	5120	<1.6	<7.0	
MBL	—	—	640	<1.0	<2.6	<1.1
MC57M		—	160	<1.7	<20	<3.3
A9HT	160	80	10,240			
JLS-V9	—	—	—	<1.8	<5.0	<2.5
BALB/3T3	—	—	—			
C3HEF1	—	—				

*Reciprocal of serum dilution producing more than 0.2 of cytotoxic index. Results represent mean values of 3 separate assays.

†ng/mg protein as determined by competition radioimmunoassay. Results represent mean values of 2 separate determinations.

‡No positive reaction at 1:10 serum dilution.

from 10–20,480. Among these positive lines, SHK of C3H, TA3Ha and TA3St of A, S2Y of ABY and SBfnHB and SBfnHD of C3H foster-nursed CBA origin were highly sensitive to these antisera. These results invariably correlated with the high concentration of MTVgp52 and p27 components in their cell extracts as detected by radioimmunoassay.

Among leukemia and sarcoma cell lines, L1210 of DBA/2 and A9HT of C3H origin were positive for MTV antigen expression at the cell surface. All mammary carcinoma lines were sensitive to anti-MuLVgp70 serum, and some of them also contained p30 components in the cell extracts. In this study, expression of MTV- and MuLV-associated antigens does not appear to relate to length of time these tumors were passaged *in vivo* before tissue culture or length of time these tumors were passaged *in vitro*. No correlation was found between antigen expression and morphology (adenocarcinoma or carcinoma).

Serological response of syngeneic or semisyngeneic mice immunized with mammary carcinomas

Out of 10 lines tested, SHF, S40C, S3W, S2Y, SBfnHA and SBfnHB were immunogenic, and preimmunization with these tumors elicited transplantation resistance in syngeneic mice. In contrast, SHK, S6C, SBfnHC and SBfnHD were not immunogenic, and grew in syngeneic hosts immunized with each tumor. Sera from the mice that had resisted multiple challenge of viable cultured SHF, S40C, S3W, S2Y, SBfnHA or SBfnHB and from the mice that had been immunized with multiple irradiated *in vivo* passaged SHF, S40C, S3W, S2Y or SBfnHA cells were examined in the complement-dependent cytotoxicity assays for antibodies to surface antigens of each immunizing tumor (Table 3).

Out of 6 immunogenic carcinoma lines, the sera of mice immunized with SHF, S3W, S2Y, SBfnHA or SBfnHB were positive for antibodies to the corresponding tumors, while the sera of mice immunized with S40C were negative. Only 6 out of 30 mice immunized with SHF produced antibodies with cytotoxic titers of 10–160. Most of the mice immunized with other tumors, S3W, S2Y, SBfnHA or SBfnHB, produced antibodies to each immunizing tumor with titers of 10-40960. No discernible correlation was found between MTV or MuLV expression and antibody production. For example, the sera of mice immunized with S2Y expressing a high amount of MTV antigens contained a high titer of cytotoxic antibodies, while the sera of mice immunized with S3W expressing very low amount of MTV antigens also contained a high level

Table 3. Complement-dependent cytotoxic antibody production in syngeneic or semisyngeneic mice hyperimmunized with cultured or in vivo *mammary carcinoma cells*

Cell lines	Host (sex)	Incidence	Antibody production (% in total)	Mean titer (range)*
Cultured cell immunization†				
SHF	C3H (f)	1/10		160
SHF	(C3H × ASW) F_1 (m)	0/5	(13)	
SHF	(A × C3H) F_1 (m)	2/9		10 (10)
S40C	ACA (f)	0/11		
S3W	ASW (f)	14/18		320 (20–40,960)
S3W	ASW (m)	3/8	(70)	40 (10–160)
S3W	(ASW × C57BL) F_1 (f)	6/7		160 (10–2560)
S2Y	ABY (f)	20/20	(100)	1280 (160–2560)
SBfnHA	CBA (f)	20/20	(100)	320 (80–2560)
SBfnHA	CBA (m)	10/10		320 (40–1280)
SBfnHA	(A × CBA) F_1 (m)	4/4		40 (20–160)
SBfnHB	CBA (f)	7/11	(70)	80 (10–1280)
In vivo tumor cell immunization†				
SHF	(A × C3H) F_1 (f)	3/6	(50)	10 (10)
S40C	ACA (f)	0/6	(0)	
S3W	ASW (f)	6/6	(100)	80 (20–320)
S2Y	(C57BL × ABY) F_1 (f)	6/6	(100)	320 (80–2560)
SBfnHA	CBA (f)	6/6	(100)	320 (40–640)

*Reciprocal of serum dilution producing more than 0.2 cytotoxic index.
†Methods of immunization and collecting sera were described in Materials and Methods.

of cytotoxic antibodies. The sera of mice immunized with SBfnHA, expressing high levels of MuLV antigens, contained a high amount of cytotoxic antibodies, while the sera of mice immunized with S40C, expressing a high amount of MuLV antigens, contained no cytotoxic antibodies. All sera obtained from normal syngeneic mice were negative for each corresponding tumor in this microcytotoxicity assay.

Expression of tumor-specific surface antigens by cultured mammary carcinomas

To determine whether antisera to syngeneic tumors contained antibodies to common or shared antigens, we tested these antisera on a panel of 13 mammary carcinomas, 9 leukemias, 4 polyoma virus-induced tumors, 6 sarcomas, 3 lines of fetal origin, and normal spleen cells derived from 3 strains of mice. Table 4 demonstrates the summarized results obtained by unabsorbed antiserum pools that were produced by the immunization with cultured carcinoma cells. All unabsorbed antisera to SHF, S3W, S2Y, SBfnHA or SBfnHB showed wide cross-reactivity with many tumors. they reacted to several carcinoma lines, and a sarcoma of C3H origin, A9HT. In addition, antisera to S3W, S2Y, SBfnHA or SBfnHB were reactive to several leukemias, and the antisera to S3W, SBfnHA or SBfnHB also reacted to polyoma virus-induced tumors, and the former two sera were reactive to methylcholanthrene-induced tumors. On the other hand, none of the antisera were cytotoxic to three lines of fetal origin and normal spleen cells of C3H, DBA/2 and C75BL origin.

In view of the wide cross-reactivity of the antisera, we performed fractionated absorption experiments. Following absorption with one of the most sensitive leukemias (RBL-5) for anti-S3W, BW5147 for anti-S2Y and ALC for anti-SBfnHA serum, the antisera no longer reacted with leukemias except for anti-S3W serum to P815 (Table 5). Anti-S2Y serum also lost activity against some mammary carcinomas, S3W, SbfnHA and SBfnHB, and anti-S3W and anti-SBfnHA sera lost activity against methylcholanthrene-induced sarcomas, MSWBS and MC57M. However, they were still reactive with several mammary carcinoma lines, and antisera to S3W and SBfnHA reacted to polyoma virus-induced tumors, SEWE, SESO, SEYFa and A9HT.

In a second absorption step, one of the most sensitive polyoma-virus induced tumor cells (SEWE for anti-S3W and SEYFa for anti-SBfnHA sera) were used until there was no reactivity left against the absorbing cells (Table 6). The residual antisera to S3W or SBfnHA showed no cytotoxicity against any of the polyoma tumors. Anti-S3W serum had also lost activity against some mammary tumors, SHF, SHK and TA3Ha and a leukemia, P815. Both absorbed antisera to S3W or SBfnHA still reacted to some other mammary carcinomas, and anti-S3W serum reacted with A9HT.

In a first absorption for anti-SBfnHB serum, RBL-5 and SEWE cells were used at the same time until there was no reactivity left against both absorbing lines (Table 6). The reason why L1210 cells were not used for this absorption in spite of the higher sensitivity of them than RBL-5 cells was because L1210 leukemia cell may contain MTV-associated antigens [20]. The residual anti-SBfnHB serum still reacted with all lines except for RBL-5 and SEWE which reacted to unabsorbed serum, although the cytotoxicity was decreased.

In a third absorption step for anti-S3W and anti-SBfnHA sera, in a second absorption step for anti-S2Y and anti-SBfnHB sera, and in a first absorption step for anti-SHF serum, one of the most sensitive other mammary carcinomas (SBfnHB for anti-SHF and anti-S3W, SBfnHD for anti-S2Y and anti-SBfnHA, and TA3Ha for anti-SBfnHB sera) was used there was no activity left against these lines (Table 7). The absorbed antisera to S3W or SBfnHA retained considerable activity against the immunizing lines, respectively, but did not react with any of the other lines tested. In contrast, the absorbed antisera to SHF, S2Y or SBfnHB failed to show any reactivity to tested lines including immunizing carcinoma lines.

We also performed similar experiments using antisera produced by immunization with irradiated *in vivo* passaged S3W, SBfnHA or S2Y. They showed almost the same specificities as those of antisera produced by immunization with cultured lines (data not shown). After absorption by leukemia, polyoma virus-induced tumor and other mammary carcinoma lines, antisera against S3W or SBfnHA retained reactivity to immunizing tumors, while antiserum against S2Y lost the activity to S2Y cells.

These results suggest that S3W and SBfnHA cells of ASW and CBA origin, respectively, carry individually distinct serologically detectable surface antigens in addition to several common antigens, while SHF, S2Y and SBfnHB carry no such serologically de-

Table 4. Specificity of unabsorbed anti-mouse mammary carcinoma sera

	Sensitivity to				
	Anti-SHF	Anti-S3W	Anti-S2Y	Anti-SBfnHA	Anti-SBfnHB
Positive lines					
Mammary carcinomas	TA3Ha (640)* SHK (160) SBfnHA (40) SBfnHB (640) SBfnHD (160) SHF (160) S40C (80) SHN (80) S3W (80) TA3St (10) S6C (160)	TA3Ha (640) SHK (20480) SBfnHB (2560) SHF (1280) S40C (640) S3W (10,240)	TA3Ha (40) SHK (1280) SBfnHA (10) SBfnHB (160) SBfnHD (640) SHN (640) S3W (20) S2Y (1280)	TA3Ha (80) SBfnHA (640) SBfnHD (640)	TA3Ha (640) SHK (1280) SBfnHA (80) SBfnHB (1280) SBfnHD (640) SHF (320) S40C (320) SHN (640) S2Y (320) TA3St (320)
Leukemias		RBL-5 (2560) L1210 (20) ALC (20) P815 (320) EL-4 (1280)	L1210 (20) ALC (10) P815 (20) EL-4 (10) BW5147 (160) YWA (40) YAC (40) YBA (40)	RBL-5 (20) ALC (160) P815 (20)	RBL-5 (160) L1210 (1280)
Polyoma virus-induced tumors		SEWE (640) SESO (320) SEYFa (160)		SEWE (10) SESO (160) SEYFa (80)	SEWE (320)
Sarcomas	A9HT (10)	A9HT (1280) MSWBS (10)	A9HT (80)	A9HT (80) MSWBS (80) MC57M (80)	A9HT (640)
Negative lines					
Mammary carcinomas	SBfnHC S2Y	SBfnHC S6C, S2Y TA3St SHN SBfnHD SBfnHA	SBfnHC S6C, S40C TA3St SHF	SBfnHC S6C, S2Y TA3St S3W, SHN SHF, SHK S40C SBfnHB	SBfnHC S6C, S3W
Leukemias	BW5147 RBL-5 L1210	BW5147 YMA, YAC YBA	RBL-5	BW5147 YWA, EL-4 L1210	BW5147
Polyomas	SEWE	SEAB	SEAB, SEWE SESO, SEYFa	SEAB	
Sarcomas	MBL	MBL, Ha2 MC57M MSB, MDAY MBK	MBL, Ha2 MC57M MSB, MDAY MSWBS	MBL, Ha2	MBL
Normal cells					
Fetal origin	JLS-V9 BALB/3T3 C3HEF1	JLS-V9 BALB/3T3 C3HEF1	JLS-V9 BALB/3T3 C3HEF1	JLS-V9 BALB/3T3 C3HEF1	JLS-V9 BALB/3T3 C3HEF1
Spleen cells	C3H, DBA/2 C57BL	C3H, DBA/2 C57BL	C3H, DBA/2 C57BL	C3H, DBA/2 C57BL	C3H, DBA/2 C57BL

*Figures in parentheses indicate reciprocal of serum dilution producing more than 0.2 cytotoxic index (mean values of 2 separate assays).

Table 5. Specificity of anti-mouse mammary carcinoma sera after absorption with leukemias

	Anti-S3W Sensitivity to RBL-5	Anti-S2Y Absorbed with BW5147	Anti-SBfnHA ALC
Positive lines			
Mammary carcinomas	TA3Ha (320) SHK (5120) SHF (640) S3W (5120) SBfnHB (640) S40C (20)	TA3Ha (20) SHK (160) SBfnHD (320) S2Y (640) SHN (320)	TA3Ha (80) SBfnHA (320) SBfnHD (640)
Leukemia	P815 (20)		
Polyomas	SEWE (320) SESO (80) SEYFa (40)		SEWE (10) SESO (40) SEYFa (80)
Sarcomas	A9HT (80)		A9HT (40)
Negative lines			
Mammary carcinomas		S3W, SBfnHA SBfnHB	
Leukemias	ALC, EL-4, RBL-5 L1210	ALC, EL-5, P815 L1210, BW5147 YWA, YAC, YBA	ALC, RBL-5, P815
Sarcomas	MSWBS	A9HT	MSWBS, MC57M

Table 6. Specificity of anti-mouse mammary carcinoma sera after absorption with leukemias and polyoma virus-induced tumors

	Anti-S3W Sensitivity to SEWE	Anti-SBfnHA Absorbed with SEYFa	Anti-SBfnHB RBL-5+SEWE
Positive lines			
Mammary carcinomas	SBfnHB (40) S40C (20) S3W (320)	TA3Ha (10) SBfnHA (80) SBfnHD (80)	TA3Ha (320) S40C (40) SBfnHB (80) SHF (80) SHK (160) SHN (80) TA3St (320) S2Y (80) SBfnHA (10) SBfnHD (40)
Leukemia			L1210 (640)
Sarcoma	A9HT (40)		A9HT (40)
Negative lines			
Mammary carcinoma	SHF, SHK TA3Ha		
Leukemias	P815		RBL-5
Polyomas	SEWE, SESO SEYFa	SEWE, SESO SEYFa	SEWE

Table 7. Specificity of anti-mouse mammary carcinoma sera after absorption with leukemias, polyoma virus-induced tumors and mammary carcinomas

	Anti-SHF SBfnHB	Anti-S3W SBfnHB	Sensitivity of Anti-S2Y Absorbed with SBfnHD	Anti-SBfnHA SBfnHD	Anti-SBfnHB TA3Ha
Positive lines					
Mammary carcinomas		S3W (160)		SBfnHA (40)	
Negative lines					
Mammary carcinomas	SHF, SHK SHN, TA3Ha TA3St, S6C S40C, S3W SBfnHA SBfnHB SBfnHD	S40C SBfnHB	SHK, SHN TA3Ha, S2Y SBfnHD	TA3Ha SBfnHD	SHF, SHK SHN, TA3Ha TA3St, S40C S2Y SBfnHA SBfnHB SBfnHD
Leukemia					L1210
Sarcoma	A9HT	A9HT			A9HT

Table 8. Antigenic specificities present on various mammary carcinomas summarized results

	Cross-reactive antigens with			
Cell lines	Leukemias and sarcomas	Polyoma tumors	Mammary carcinomas and a C3H sarcoma	Unique antigens
SHF	−	−	+	−
S3W	+	+	+	+
S2Y	+	−	+	−
SBfnHA	+	+	+	+
SBfnHB	+	+	+	−

tectable distinct antigens but only common antigens with other mammary carcinomas, leukemias, polyoma virus-induced tumors and/or other sarcomas (Table 8).

DISCUSSION

The present study demonstrates that most of 13 established culture lines derived from spontaneous and virus-induced mammary carcinomas of six different mouse strains express high amount of the major MTV envelope antigen gp52 at the cell surface. These results confirm previous reports by other authors [1, 3, 4]. Further, at least six lines from C3H, A, ABY and CBA origin contained large amounts of two MTV major structural proteins, gp52 and p27, in the cell extracts. Electronmicroscopic findings reported separately show that these cultured lines are of mammary carcinoma origin [16]. The mammary carcinoma lines also showed sensitivity to anti-MuLV major structural components gp70 and some of them contained p30 in the cell extracts. Concomitant expression of MTV and MuLV has previously been reported [1–4].

Five out of ten lines induced antibodies after hyperimmunization of syngeneic or semisyngeneic mice, as detected by complement-dependent cytotoxicity. MTV expression was unrelated to the inducibility of antibodies. Not only high MTV producer lines, S2Y and SBfnHB, but also low MTV producer lines, SHF, S3W and SBfnHA induced antibody. Expression of MuLV-associated antigens in these lines did not correlate with the inducibility of syngeneic antibodies.

The antibody production in syngeneic or semisyngeneic hosts permitted us to serologically analyse tumor-associated surface antigens of mouse mammary carcinomas. All unabsorbed antisera to five different mammary carcinomas invariably showed multiple specificity. They reacted not only with other mammary carcinomas, but also with leukemias, polyoma virus-induced tumors, and other sarcomas. Similar multispecificity were reported in other experimental tumor systems [21, 22] and in human tumor systems [23, 24].

Stück *et al.* [25] reported that there was a cross-reactive antigen (ML) between mammary tissue and mammary tumors of mice infected with MTV and certain leukemias of DBA/2 origin. The L1210 cells express the ML antigen on the cell surface [20]. It was not found in the mammary tissues and tumors

of mice from uninfected strains and in the leukemias of other strains except for GR strains [6, 26]. Our results showed that syngeneic antisera to four different mammary carcinomas reacted not only to DBA/2 leukemias, L1210 and p815, but also to leukemias from AKR, A, ASW, CBA or C57BL mice. These results suggest that the cross-reactive antigens between our mammary carcinomas and leukemias are not identical to the ML antigen. Brown *et al.* [27] found that chemically induced sarcomas expressing MuLVgp70 at the cell surface induced the formation of antibodies specific for the MuLV viral envelope antigen gp70. The cross-reactive antigen in our studies could be MuLV70 antigen, because mammary carcinomas, leukemias and sarcomas expressed the antigen. However, there were several leukemias which were sensitive to rabbit anti-gp70 serum but insensitive to anti-mammary carcinoma sera, suggesting that the cross-reactive antigen is not group-specific gp70 antigen.

Antisera against three different mammary carcinomas also reacted to polyoma virus-induced tumors in the present studies. This cross-reactivity may be related to polyoma virus-induced antigens, in view of the ubiquity of the virus in many mouse strains. The antisera to mammary carcinomas after absorption with leukemias and/or polyoma virus-induced tumors still reacted to other mammary carcinomas in the present studies. This cross-reactivity disappeared after absorption with one of other mammary carcinomas, suggesting that it is directed against mammary carcinoma-associated antigen(s), presumably MTV-associated antigen(s).

Further, our present results showed that antisera to at least two different mouse mammary carcinomas still remained specific to the respective immunizing tumors after absorption with cross-reactive leukemias, polyoma virus-induced tumors and other mammary carcinomas. These results serologically confirmed the previous reports by others [7, 11–15] that some mammary carcinomas have individually distinct antigens in addition to common antigens with other mammary carcinomas. There has been some speculation that the unique antigens of chemically induced and other tumors might represent rearranged antigens of the H-2 complex [28], derepressed fetal or alloantigen [29], antigens induced by spontaneous point mutation or antigens associated with epigenetic errors in membrane synthesis. Certain similarities between chemically or virally induced tumors, especially the presence of individually distinct antigens, suggest that a common mechanism may be operative which produces these antigens during carcinogenesis by both types of oncogenic agents. However, one can not exclude the possibility that the unique antigens might be virus-induced. Two types of MTV are present in inbred strains of mice, the endogenous and exogenous MTVs [30]. It has also been shown that there are type-specific determinants on the gp52 molecule of MTVs from C3H, GR and RIII mice [31]. Therefore, the distinct antigens could arise from the presence in these tumors of unrecognized variants of MTV, resulting in the formation of new immunizing structure on the cell surface.

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