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C3H/HeN Mammary Tumor-Bearing Mice Develop Type-Specific Neutralizing Antibodies and Group-Specific Precipitating Antibodies for the Mouse Mammary Tumor Virus

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The development of mouse mammary tumor virus (MMTV)-neutralizing antibodies in various strains of mice was measured by their ability to neutralize the focus-forming capacity of a Kirsten sarcoma virus (C3H MMTV) pseudotype containing the MMTV envelope glycoprotein gp52. C3H/HeN, but not GR/N and RIII, mammary tumor-bearing mice were found to develop neutralizing antibodies to this pseudotype. In addition, non-tumor-bearing C3H/HeN, GR/N, RIII, NIH Swiss, C57BL/6, and BALB/c mice and 13 feral mice were also negative for neutralizing antibodies. The neutralization was immunoglobulin G mediated, and the antibodies of C3H/HeN mammary tumor-bearing mice were type specific and capable of distinguishing C3H and GR/N MMTVs from RIII and C3H/HeNf MMTVs. Precipitating antibodies were detected in sera from RIII and GR/N tumor-bearing mice, GR/N non-tumor-bearing mice, and six of the feral mice, although these same sera did not neutralize the Kirsten sarcoma virus (C3H MMTV) pseudotype. The results of this study and of a previous study demonstrate that C3H/HeN mammary tumor-bearing mice develop three functionally distinct antibody populations: (i) group-specific virus-precipitating antibodies; (ii) type-specific virus-neutralizing antibodies; and (iii) type-specific cytotoxic antibodies.

Mouse strains with both high (C3H/HeN, GR/N, and RIII) and moderate (C3H/HeNf) incidences of mammary tumors develop a humoral immune response to the mouse mammary tumor virus (MMTV) (6, 9, 10, 13). These mice develop cytotoxic (8) and precipitating (2, 11) antibodies, both of which have been demonstrated in vitro for MMTV-infected cells and MMTV, respectively. The cytotoxic antibodies have been shown to be type specific, can distinguish C3H and GR/N MMTVs from RIII MMTV, and appear to be directed against the major external MMTV glycoprotein gp52 (15). The precipitating antibodies appear to be group specific and can precipitate C3H, GR/N, and RIII MMTVs; however, the nature of the MMTV target antigen is unclear (8).

Although serum from mice precipitated MMTV, it was not known whether this interaction would neutralize the infectivity of the virus. Until recently, neutralization of MMTV had been measured by an in vivo assay based on the inhibition of MMTV-induced hyperplastic nodules in mammary glands of mice (5). This test required 5 months for completion, and neutralization was observed only with sera from adult mice hyperimmunized with MMTV. However, with the development of MMTV pseudotypes which contain MMTV gp52 as an envelope glycoprotein, neutralization could be measured in vitro. Heterologous, hyperimmune anti-MMTV serum inhibited the lytic capacity of an MMTV pseudotype of vesicular stomatitis virus (17) and the transforming activity of an MMTV pseudotype of Kirsten sarcoma virus (KiSV) (16). We tested the ability of natural mouse sera to neutralize the focus-forming capacity of KiSV (C3H MMTV). The results of these studies demonstrated that C3H/HeN, but not GR/N or RIII, mammary tumor-bearing mice developed neutralizing antibodies. These antibodies were type specific and capable of distinguishing C3H and GR/N MMTVs from RIII and C3H/HeNf MMTVs. All of these sera were shown to have precipitating antibodies to C3H MMTV. The results of this study and of a previous study (8) demonstrated that C3H/HeN mammary tumor-bearing mice develop three functionally distinct antibody populations.

MATERIALS AND METHODS

Cells and viruses. Fischer rat embryo (FRE) cells were propagated in Eagle-modified minimal essential medium with Earle salts (EMEM) containing 5% fetal

calf serum. C3H/Crgl (C3H), GR/N, and RIII MMTVs and Gross murine leukemia virus (G-MuLV) were all obtained through the Viral Resources Laboratory at the Frederick Cancer Research Center. A cloned cell line producing high levels of MMTV was derived from a spontaneous mammary tumor occurring in a C3H/HeNf mouse and was used as the source for C3Hf clone 14 MMTV (4). KiSV (C3H MMTV) was propagated as previously described (15).

Animals and sera. NIH Swiss, BALB/c, C57BL/6, GR/N, C3H/HeN, and C3H/HeNf NIH Swiss female mice were obtained from the Animal Production Facility at the Frederick Cancer Research Center. The spontaneous mammary tumor incidence in female breeders of these strains has been reported previously (11). RIII mice were obtained from A. S. Dion, Institute for Medical Research, Camden, N.J. All normal and mammary tumor-bearing mice used for this study were 6 to 8 months old. Blood was obtained from the retro-orbital plexus and allowed to clot at 4°C overnight. Sera were separated by centrifugation, aliquoted, and stored at −20°C until used. In some cases, sera from mice of the same strain, age, and tumor status were pooled. Sera from feral mice and feral mice infected with C3H/HeJ MMTV by foster nursing on C3H/HeJ mothers were kindly provided by M. Gardner, University of Southern California, Los Angeles. These feral mice were trapped in the Lake Casitas and Bouquet Canyon areas of southern California.

Rabbit antisera directed against MMTV and MMTV proteins gp52, gp36, p27, p14, and p10 were prepared and characterized as previously described (3). The anti-MMTV serum was highly specific for MMTV and exhibited no detectable cross-reactivity with various MuLV's, including ecotropic Rauscher MuLV (R-MuLV), xenotropic BALB-2 virus, the dual-tropic mink cell focus virus of AKR mice, and the amphotropic MuLV of feral mice (14). Goat anti-R-MuLV gp70 serum was kindly provided by S. Oroszlan, Frederick Cancer Research Center. This antiserum possessed precipitating and neutralizing antibodies against a variety of MuLV's, including R-MuLV, AKR ecotropic virus, xenotropic BALB-2 virus, mink cell focus virus, and amphotropic MuLV. A monoclonal antibody (VI P3C5) to C3H MMTV gp52 was prepared by the methods previously described for producing monoclonal antibodies to MuLV p15(E) (12). Ascites fluids from a BALB/c mouse inoculated with these hybridoma cells were used for the neutralization study. This antibody is a mouse immunoglobulin G2a (IgG2a) which precipitates only MMTV gp52 (R. J. Massey, L. O. Arthur, R. C. Nowinski, and G. Schochetman, manuscript in preparation). The titer (reciprocal of the highest dilution reacting with virus) for this monoclonal antibody, as measured by an iodinated protein A assay (12), was 10^6 for C3H MMTV.

Serum fractionation. A C3H/HeN tumor bearer serum pool was fractionated by centrifuging 1.0 ml of serum on a 5 to 15% (wt/wt) gradient as previously described (1). The gradient was fractionated, and each fraction (1 ml) was dialyzed against Eagle-modified minimal essential medium with Earle salts. Each fraction was then tested for the following: (i) gp52 content in a competitive radioimmunoassay (1); (ii) IgG concentration by radial immunodiffusion; and (iii) neutralizing capacity.

Neutralization of focus formation. Antisera were heat inactivated at 56°C for 30 min before use. Stock virus supernatants of KiSV (C3H MMTV) were diluted to contain 250 foci in 0.5 ml. Then, 0.5-ml dilutions of antisera were mixed with 0.5 ml of virus and incubated for 30 min at 37°C. Virus-antiserum mixtures (0.4 ml) were added to duplicate 60-mm dishes containing indicator cells (5×10^5 cells per dish) that had been seeded 24 h before in medium containing 2 μg of polybrene per ml. The medium was removed before the addition of the virus-antiserum mixture. Incubation was continued for 1 h at 37°C. The dishes were then overlaid with 4 ml of Eagle-modified minimal essential medium with Earle salts containing 5% fetal calf serum. Foci appeared between 10 and 14 days later, at which time the cells were fixed and stained, and the foci were counted. The number of foci induced in the presence of antiserum (V_n) and the number of foci induced in the absence of antiserum (V_0) were used to calculate the effect of the serum on viral infectivity as previously described (7). The percent reduction was calculated as $100[(V_0 - V_n)/V_0]$ instead of the surviving fraction, V_n/V_0, as previously described (7).

Serum adsorptions. To each 0.1 ml of undiluted C3H/HeN tumor bearer serum pool no. 2, 0.9 ml of each virus (50 μg) was added, and the resulting mixtures were incubated at 37°C for 90 min, followed by incubation at 4°C overnight. The adsorbed sera were centrifuged at 100,000 × *g* for 1 h to remove immune complexes. The supernatants were then tested in the neutralization assay.

Radioimmunoprecipitation assays. The radioimmunoprecipitation assay was performed with ^{125}I-labeled MMTV as previously described (2). Briefly, antibodies to MMTV were detected by incubating 10,000 cpm of radiolabeled antigen in 100 μl of radioimmunoprecipitation buffer (0.01 M Tris-hydrochloride [pH 7.4], 0.01 M NaCl, 0.001 M EDTA) with 100 μl of diluted mouse serum for 2 h at 37°C and overnight at 4°C. *Staphylococcus aureus* (Cowan I strain) was added to each tube to enhance the precipitation of antigen-antibody complexes, and the tubes were incubated for 15 min at room temperature. The pellets were collected at 1,500 × *g* for 30 min and then washed with buffer. Radioactivity remaining in the pellets was determined with a Searle gamma counter.

RESULTS

Neutralization of KiSV (C3H MMTV) pseudotype by natural mouse sera. The KiSV (C3H MMTV) pseudotype is a transforming virus containing the transforming gene of KiSV and the envelope glycoprotein of C3H MMTV, gp52 (16). Transformation was quantitated by focus formation which followed one-hit kinetics, indicating that all of the information required for transformation resided in a single particle (16). The target antigen for the neutralization of transformation was gp52 because only rabbit anti-gp52 serum and a monoclonal anti-

body (VI P3C5) against C3H MMTV gp52 could neutralize the pseudotype (Table 1). In contrast, antisera against the other MMTV proteins, gp36, p27, p14, and p10, were negative, as was a broadly reactive anti-R-MuLV gp70 serum. The anti-gp70 serum, however, could completely neutralize equivalent amounts of ecotropic, xenotropic, and amphotropic strains of MuLV.

An important application of the pseudotype was to determine whether natural mouse sera contained MMTV-neutralizing antibodies as measured by their ability to inhibit the focus-forming capacity of KiSV (C3H MMTV). Of all the mouse sera tested, only sera from mammary tumor-bearing C3H/HeN mice contained MMTV-neutralizing antibodies (Table 2). Table 2 shows the variation in neutralization titers for two C3H/HeN tumor bearer serum pools. Complete titration curves for some of the antisera are shown in Fig. 1. Sera from non-tumor-bearing C3H/HeN animals were negative, as were sera from the low mammary tumor incidence NIH Swiss, BALB/c, and C57BL/6 mice. An important observation was that sera from high-incidence mammary tumor-bearing mice, RIII and GR/N, contained no detectable neutralizing antibodies against KiSV (C3H MMTV), even though these sera contained high MMTV-precipitating antibody titers.

Sera from outbred feral mice were also assayed for MMTV-neutralizing antibodies. Table 3 shows that none of the 13 sera tested exhibited any significant levels of neutralizing antibodies. These included four non-tumor-bearing mice foster nursed on C3H/HeJ mothers to introduce the exogenous MMTV. Similar to the results obtained with RIII and GR/N mice, there was no correlation between precipitating antibody titers and neutralizing antibody titers.

TABLE 1. *Ability of antisera directed against individual MMTV proteins to neutralize KiSV (C3H MMTV) pseudotype transformation*

Antiserum	RIP titer[a]	% Reduction of focus formation[b] at following antiserum dilution:			
		1:8	1:32	1:64	1:128
Anti-MMTV	1:51,200	100	100	100	100
Anti-gp52	1:1,600	95	72	48	27
Anti-gp36	1:1,600	0	0	0	0
Anti-p27	1:1,600	0	0	0	0
Anti-p14	1:3,200	0	0	0	0
Anti-p10	1:3,200	0	0	0	0
Anti-MuLV gp70	$1:10^5$	0	0	0	0
VI P3C5[c]	$1:10^4$	100	100	100	100

[a] Antiserum dilution which precipitated 50% of the radioactivity of iodinated homologous antigen. RIP, Radioimmunoprecipitation.

[b] Percent reduction of focus formation = [1 − (number of foci induced in the presence of antiserum/number of foci induced in the absence of antiserum)] × 100.

[c] IgG2a monoclonal antibody directed against C3H MMTV gp52. The neutralization titer of this monoclonal antibody, based on the highest dilution yielding a 10% reduction in the number of foci, was $>10^5$.

TABLE 2. *Assay of sera from normal and mammary tumor-bearing mice to neutralize KiSV (C3H MMTV) pseudotype transformation*

Mouse strain[a]	RIP titer[b]	% Reduction of focus formation[c] at following antiserum dilution:			
		1:8	1:32	1:64	1:256
Tumor bearing					
C3H/HeN (pool no. 1)	1:640	76	18	0	0
C3H/HeN (pool no. 2)	1:1,280	66	32	19	0
GR/N	1:640	0	0	0	0
RIII	1:320	0	0	0	0
Non-tumor bearing					
C3H/HeN	<1:20	8	0	0	0
C3H/HeNf NIH Swiss	<1:20	0	0	0	0
GR/N	1:640	0	0	0	0
C57BL/6	<1:20	0	0	0	0
BALB/c	<1:20	0	0	0	0
NIH Swiss	<1:20	0	0	0	0
NIH Swiss + 40 μg of C3H MMTV	<1:20	0	0	0	0

[a] Sera were pooled from six mice for each mouse strain tested.

[b] Antiserum dilution which precipitated 50% of the ^{125}I-labeled C3H MMTV. RIP, Radioimmunoprecipitation.

[c] See Table 1 for calculation of percent reduction of focus formation.

Mediation of neutralization by IgG antibodies. To determine whether the neutralization was mediated by antibodies, heat-inactivated serum pool no. 2 from C3H/HeN tumor-bearing mice was fractionated by velocity sedimentation on a 5 to 15% (wt/wt) sucrose gradient. Individual gradient fractions were analyzed for gp52 content with a gp52 radioimmunoassay and for IgG concentration with a radial immunodiffusion assay. Each fraction was also assayed for its capacity to neutralize the KiSV (C3H MMTV) pseudotype. Figure 2 shows that the peak neutralizing activity coincided with the peak IgG concentration but not with MMTV or free gp52 or where IgM would be expected to sediment. This demonstrated that neutralization was mediated by IgG and excluded the possibility that MMTV, free gp52, or some soluble factor was involved in focus reduction. The possibility that focus reduction was the result of interference by free MMTV in the mouse serum was further excluded by the fact that NIH Swiss sera containing C3H MMTV, which was added to levels comparable to levels found in C3H/HeN tumor bearer sera, did not neutralize KiSV (C3H MMTV) (Table 2).

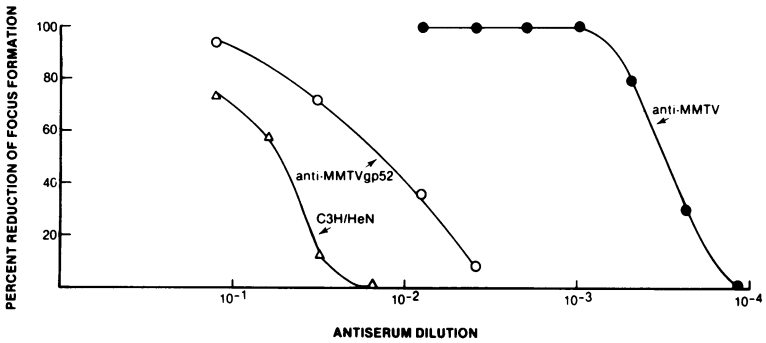

FIG. 1. *Neutralization of the transformation by the KiSV (C3H MMTV) pseudotype by various antisera. FRE cells were used as indicator cells for transformation, and neutralization was assayed as described in the text. The C3H/HeN serum was from a mammary tumor-bearing animal.*

TABLE 3. *Assay of sera from feral mice to neutralize KiSV (C3H MMTV) pseudotype transformation*

Mouse	RIP titer[a]	Neutralization titer[b]
C3H/HeN	1:1,280	1:64
Feral mice foster nursed on C3H/HeJ mice		
27666B	1:320	<1:10
27673A	>1:1,280	<1:10
27672A	<1:5	<1:10
27677A	<1:5	<1:10
Feral mice not foster nursed[c]		
18841 (LC)	1:160	<1:20
20815 (BC)	1:1,280	<1:40
22324 (BC)	1:320	<1:20
22840 (BC)	1:320	<1:20
18800 (LC)	<1:5	<1:20
18851 (LC)	<1:5	<1:20
20776 (BC)	<1:5	<1:20
23947 (LC)	<1:5	<1:20
24095 (LC)	<1:5	<1:20

[a] Dilution of antiserum yielding a 20% precipitation of radiolabeled MMTV. RIP, Radioimmunoprecipitation.

[b] Dilution of antiserum yielding a 10% reduction in the number of foci.

[c] LC, Lake Casitas area; BC, Bouquet Canyon area.

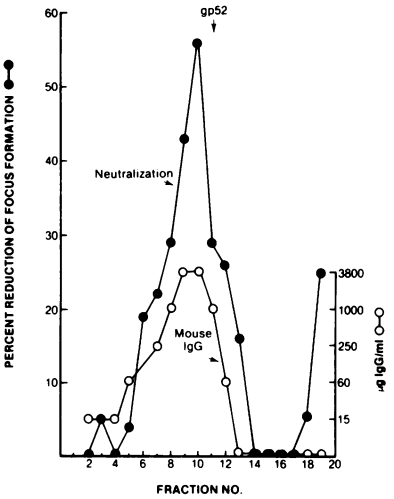

FIG. 2. *Fractionation of C3H/HeN tumor-bearer serum pool no. 2. The serum pool was fractionated on a 5 to 15% (wt/wt) sucrose gradient as described in the text. Fractions (1 ml each) were collected and assayed for gp52 content, mouse IgG concentration, and neutralizing capacity. The peak gp52 concentration (top arrow) was 84 μg/ml.*

Demonstration of type-specific neutralizing antibodies in the sera of C3H/HeN mammary tumor-bearing mice. The inability of sera from the RIII and GR/N tumor-bearing mice to neutralize focus formation by KiSV (C3H MMTV) is presumably due to the development of neutralizing antibodies specific for their homologous virus. Because pseudotypes containing envelope gp52's of RIII and GR/N MMTVs were not available, the type specificity of neutralizing antibodies was determined by the ability of various strains of MMTV to adsorb the neutralizing antibodies of sera from C3H/HeN tumor-bearing mice. The serum was diluted approximately 1:20 to yield 70% neutralization. The C3H/HeN tumor bearer serum pool no. 2 was extensively adsorbed with C3H, GR/N, RIII, or C3Hf clone 14 MMTV, and the adsorbed sera were then tested for their ability to neutralize the focus-forming capacity of the KiSV (C3H MMTV) pseudotype (Table 4). When the data were normalized to 0% inhibition for the unadsorbed sera, it was clear that C3H and GR/N MMTVs could completely adsorb the neutralizing antibodies. In contrast, RIII MMTV and C3Hf clone 14 MMTVs were

TABLE 4. *Type specificity of neutralizing antibodies in sera of C3H/HeN mammary tumor-bearing mice*

Serum adsorbed[a] with:	Avg no.[b] of foci per plate	% Inhibition of neutralization[c]
Unadsorbed	17	0
C3H MMTV	64	95
GR/N MMTV	67	100
C3Hf clone 14 MMTV	35	36
RIII MMTV	33	32
G-MuLV	12	0

[a] Serum was diluted 1:10 to yield ~70% neutralization. Each adsorption was performed with 50 μg of virus for 30 min at 37°C.

[b] The average number of foci on control plates which were not treated with antiserum was 67.

[c] Percent inhibition of neutralization = [1 − (percent reduction of focus formation with adsorbed serum/percent reduction of focus formation with unadsorbed serum)] × 100.

able to adsorb a maximum of only one-third of the neutralizing antibodies. Therefore, the neutralizing antibodies were able to distinguish C3H/HeN and GR/N MMTVs from RIII and C3Hf clone 14 MMTVs. The inability of G-MuLV to adsorb any of the neutralizing activity demonstrated that the neutralizing antibodies were directed against MMTV.

DISCUSSION

The results presented here provide the first demonstration that C3H/HeN mice with spontaneously arising mammary tumors develop IgG neutralizing antibodies directed against MMTV. It was also demonstrated that the neutralizing antibodies of C3H/HeN mice are type specific and are capable of distinguishing C3H and GR/N MMTVs from RIII and C3H/HeNf MMTVs. The results for the type specificity of the neutralizing antibodies agree with our results for the type specificity of cytotoxic antibodies (15). We have been unable to directly demonstrate that the neutralizing antibodies of the mouse are directed against MMTV gp52. However, the ability of only anti-gp52 serum and monoclonal anti-C3H MMTV gp52 antibody, but not antisera against MMTV gp36, p27, p14, and p10, to neutralize the pseudotype indicates that gp52 is a target antigen for neutralization. The presence of type-specific determinants on gp52 is further supported by the demonstration that C3H/HeN and GR/N MMTVs are able to compete completely in a type-specific radioimmunoassay for C3H MMTV gp52 with heterologous antisera (4, 15). In this assay, RIII and C3H/HeNf MMTVs yielded no competition. The results of these various studies indicate that C3H and GR/N MMTV gp52's contain shared type-specific determinants which are not present on the gp52's of RIII and C3H/HeNf MMTVs.

The ability to find mice with high MMTV-precipitating antibody titers in the absence of detectable neutralizing antibody titers (Table 2) indicates that they may represent two distinct antibody populations. The potential existence of functionally distinct antibody populations within an animal has also been observed for precipitating and cytotoxic antibodies in mammary tumor-bearing mice (8). Thus, it would appear that mammary tumor-bearing mice develop at least three functionally distinct antibody populations: (i) virus-precipitating antibodies which are group specific; (ii) gp52 cytotoxic antibodies which are type specific; and (iii) virus-neutralizing antibodies which are also type specific. The latter two exhibit the same patterns of type specificity, as demonstrated by their ability to distinguish C3H and GR/N MMTVs from RIII and C3H/HeNf MMTVs.

The ability to measure neutralization of MMTV in vitro, together with the ability to measure precipitating and cytotoxic antibodies, should enable one to approach the question of the significance of these responses in vivo in the development of mammary tumors induced by MMTV.

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