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ANTIGENIC CHARACTERISTICS OF METASTATIC AND NONMETASTATIC RAT MAMMARY ADENOCARCINOMA LINES DURING PASSAGE IN NUDE MICE

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ABSTRACT: Allogeneic and xenogeneic tumor grafts are usually accepted by athymic nude mice, and such grafts grow only at the site of inoculation. The spontaneously metastasizing rat mammary tumor, TMT081, however, fails to grow in nude mice. The nonmetastasizing tumor, MT100, grows progressively in nude mice, often accompanied by hematogenous spread in lung and liver. To understand the basis for such changes in the biological behavior of these tumors, the antigenic profiles of these tumors were investigated as they grew in rats and nude mice. The nonmetastatic MT100 tumor of Wister Furth rats expressed two antigens during its propagation in rats but expressed only one of these two antigens, when the tumor was passaged in nude mice. This loss of the tumor-associated antigen was reversible, and the antigen could be detected again after retransplantation of the tumor from the nude mice into rats. Furthermore, the unexpressed antigen evoked an antibody response in nude mice, which probably masked or modulated the expression of this antigen. Since no such antibody was induced in rats, the antigen reappeared, when the tumor was grafted back into rats. Along with such changes, the MT100 tumor acquired some features of metastatic tumors and spread to other organs. The rat metastatic tumor TMT081, on the other hand, evoked no detectable humoral response in nude mice and was also rejected. In contrast, the TMT081 tumor elicited a humoral response in rats detectable as soluble immune complexes in the presence of a large excess of MTA, an organ-specific antigen. Thus, the host's immune status appears to greatly influence the expression of the antigenic characteristics of the tumors and to possibly influence the biological behavior of these tumors as well.

INTRODUCTION

The acquisition or loss of a tumor phenotype has been observed in many laboratories (Ghosh and Bankert, 1984; Nicolson, 1984; Schirrmacher, 1980). This often occurs during progression of the tumor *in vivo* (Chow and Greenberg, 1980; Ghosh and Bankert, 1984). The host's response to the tumor has often been implicated in the generation of tumor heterogeneity (Nicolson, 1984; Schirrmacher, 1980; Chow and Greenberg, 1980; Ghosh, *et al.,* 1987, 1990; Ghosh and Bankert, 1989). The question of whether the phenotypic variations in tumor cells occur because of inherent instability and/or due to interaction with host factors is not clear. However, various immunologic mechanisms may effectively cause changes in tumor cell phenotype, thus allowing tumors to escape the host's immune recognition (Ghosh and Bankert, 1989; Ghosh, *et al.,* 1987, 1990; Jones, *et al.,* 1986; Miller, *et al.,* 1979). Recently, the Thy1+Lyt1+L3T4+Lyt2-lymphocytes specific for a tumor-associated cell surface antigen have been shown to be responsible for the generation or selection of antigen-loss variants of a B cell tumor during its natural progression in tumor-

bearing mice (Ghosh and Bankert, 1989; Ghosh, *et al.,* 1987, 1990).

The influence of the host's immune system on the metastatic potential of a tumor has been intriguing immunologists for some time (Nicolson, 1984; Kim, *et al.,* 1982). Whether this is intrinsic to a tumor or could be acquired as a result of host-tumor interactions is not currently understood.

The graft of metastatic TMT081 tumor in athymic nude mice is rejected outright or accepted only for a short period of time with no sign of metastasis (Kim, *et al.,* 1982). On the other hand, the nonmetastasizing MT100 tumor is readily accepted by nude mice and grows rapidly. The latter produces a large tumor and often induces hematogenous metastasis in the lungs and liver.

In order to understand the biological basis for the reversal of metastatic behavior in these tumors, the phenotypic changes that occur during the growth of these tumors in syngeneic rats (Ghosh, *et al.,* 1983a) as well as in immunocompromised hosts such as nude mice were studied. The growth of MT100 in athymic nude mice causes a reversible shift in the phenotypic expression of this tumor, but no detectable changes occur in the phenotype of TMT081, which is eventually rejected by the nude mouse host. The nonmetastatic mammary carcinoma, MT100, has been shown to evoke humoral response in the nude host but apparently not in rats. The MT100 tumor also acquires a metastatic phenotype, when it grows in nude mice (Kim, *et al.,* 1982). On the other hand, histologically matched TMT081 evokes no detectable antibody response in the nude mice but does evoke a response in rats in which it is highly metastatic (Ghosh and Roholt, 1984).

Thus, it appears that the antigenicity of a tumor may contribute significantly to the metastatic phenotype of a tumor. Furthermore, the presence or absence of an intact thymus seems to be of significance in the elicitation of specific antibody response to these rat tumors.

MATERIALS AND METHODS

Tumors. Transplantable nonmetastatic rat mammary adenocarcinomas, MT100 and spontaneously metastasizing TMT081, were originally induced by 3-methylcholanthrene (Ghosh, *et al.,* 1979; Kim, 1970). The tumors were maintained by serial transplantation in intact male or female WF rats. A cultured cell line of TMT081 designated as TMT081-MS and a cultured cell line of MT100 designated as MT100-TC were also used (Ghosh, *et al.,* 1983b). The biological and immunochemical characteristics of these tumors have been extensively studied (Ghosh, *et al.,* 1979). Briefly, subcutaneous inoculations of metastatic TMT081 in the ingiunal mammary fat pad of WF rats resulted in the growth of the tumor not only at the site of transplantation but also in the lymph nodes, lungs, bone marrow, and kidneys. A similar graft of MT100, the nonmetastatic rat tumor, into WF rats would grow expansively only at the site of transplantation but never spread to other tissues (Kim, *et al.,* 1982). However, both these transplantable tumors are highly malignant and eventually kill the rats (Kim, *et al.,* 1982). The polyoma-virus-induced fibrosarcoma, PW739, was used as a nonmammary control tumor (Ghosh, *et. al.,* 1978).

Athymic nude mice. Two- to four-month old athymic nude mice of BALB/c background and of both sexes were used. Finely minced tumors in tissue culture

Table1. Level of mammary tumors-associated antigen (MTA) in tumor cell homogenates and in the sera of tumor-bearing animals.

Animals[1]	Tumor[2]	Type[3]	Feature[4]	MTA[5]	
				Unit / g Tumor	Unit / ml Sera
Rats	MT100	Solid	NM	490±75	< 2
	MT100-TC	Cell line	NM	900±100	< 2
	TMTO81	Solid	M	26000±1500	26±4
	TMTO81-MS	Cell line	M	12045±943	22±1.8
Nude mice	MT100	Solid	M	167±92	< 2
	MT100-TC	Cell line	M	121±25	< 2
	TMTO81	Solid	NM	15435±894	45±6.5
	TMTO81-MS	Cell line	NM	6000±540	15±3.3

[1] Five rats and four nude mice were used for each tumor.

[2] Transplantable mammary tumors of WF rats were implanted with a 13 gauge trocar.

[3] Refers to tumors that grew out as solid tumors *in vivo* as well as those that were derived from the solid tumors and grew as cell lines *in vitro* (see Ghosh, *et al.,* 1983b).

[4] NM indicates nonmetastatic and M stands for metastatic tumors. Metastatic tumors spread and grow in the lymph nodes, lung, liver, bone, spleen, and occasionally kidney. Nonmetastatic tumors grow lethally at the site of inoculation.

[5] One unit is the amount of antigen needed to produce 50% inhibition of the specific binding of radiolabeled antigen by antibody (Kim, 1970). Sensitivity limit of the assay is about 2 units/ ml. Duplicate samples from each animal were analyzed, and the values represent the means ± the standard deviation.

medium 199 containing 1% penicillin-streptomycin were injected subcutaneously into the right flank of the nude mice and watched for growth (Kim, *et al.,* 1982). The metastatic rat tumor, TMT081, grows only at the site of grafting in nude mice for a few weeks. It does not metastasize in the nude hosts; that is, the TMT081 tumor does not spread to other sites or organs (Kim, *et al.,* 1982). The nonmetastatic MT100 tumor, on the other hand, grows to a very large size in the nude mice during the same period and often spreads to the lungs and liver (Kim, *et al.,* 1982).

Preparation of rabbit antisera against TMT081 and MT100 tumors. The details of the production of rabbit antisera against rat tumors have been described elsewhere (Ghosh, *et al.,* 1979, 1983b). Briefly, finely chopped tumors were strained and homogenized in pH 8.0 borate buffer, and the post-nuclear membrane fractions were prepared by differential centrifugation. For each tumor, the membrane suspension (about 6 mg protein) emulsified in complete Freund's adjuvant was injected indermally along the backs and into the hind footpads of six rabbits. Three similar injections of the membrane fractions in Freund's complete adjuvant were given intra-

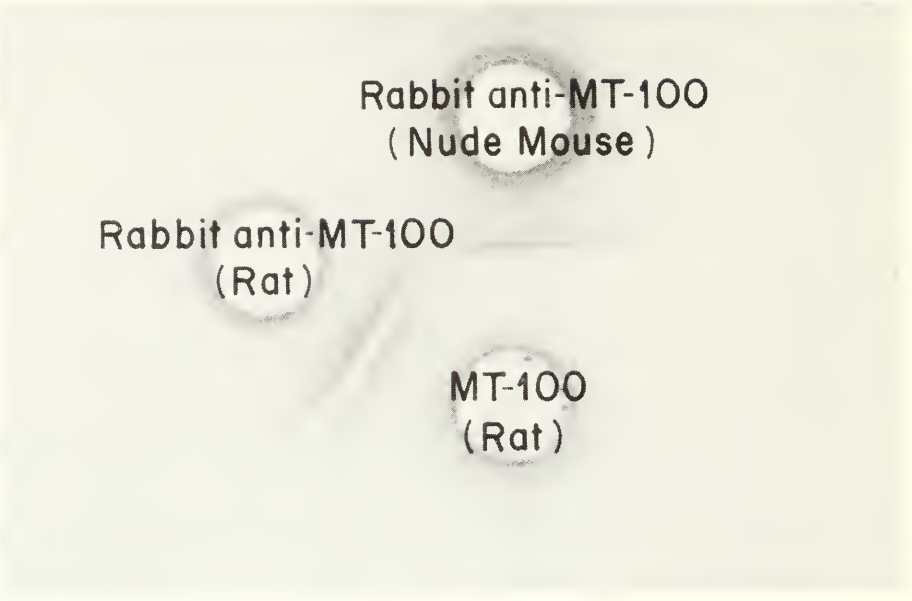


Figure 1. Immunodiffusion in agarose gel demonstrating antigenic modulation in the MT 100 tumor. It shows that rabbit antisera to MT100 (from nude mouse) recognize only one of the two antigens in the original rat MT100 tumor homogenate.

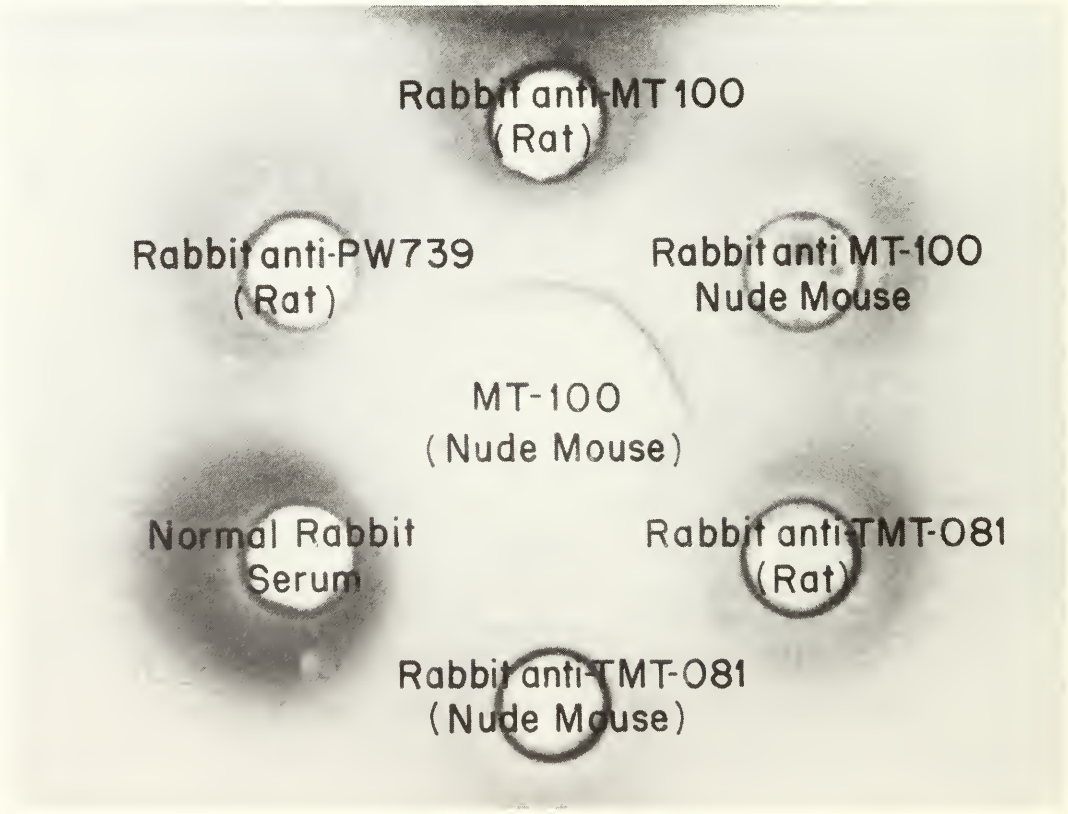


Figure 2. Immunodiffusion experiment demonstrating that rabbit anti-MT100 from both rats and nude mice recognize specifically only one and same antigen in the homogenate of MT100 (nude mice).

dermally at four-week intervals. Rabbits were bled once every week, beginning one week after the second injection. The sera were heat inactivated for 30 minutes at 56° C and kept frozen until used. The antisera used here were from rabbit number 7146 in the case of TMT081 and number 7066 in the case of MT100.

Rabbit antisera against nude mouse-derived rat mammary tumors were obtained by immunization of New Zealand White rabbits with tumor membrane preparations. For each rat tumor, two rabbits were immunized. The procedure was essentially the same as described above. Antisera from one rabbit for each tumor, namely, No. 7444 for MT100 (nude mouse) and No. 7463 for TMT081 (nude mouse), were used in these experiments.

These antisera were adequately absorbed with normal rat and nude mouse tissues consisting of liver, spleen, and muscle. In immunodiffusion as well as in radioimmunoassay (RIA), these antisera reagents did not show any reactivity to normal rat or nude mouse tissues. Before use, all these absorbed antisera were heat-inactivated at 56° C for 30 minutes and mixed with normal rat serum (10:1).

Immunodiffusion. This was done on microscope slides coated with 1.2% agarose (Fischer Scientific Co.) in veronal buffer pH 8.6 containing 0.3% polyethylene glycol. Diffusion was allowed to proceed for 24 hours at room temperature in a moist chamber.

Radioimmunoassay (RIA) to determine common TMT081-associated mammary tissue-specific antigen (MTA). This procedure was described in a previous publication (Ghosh, *et al.,* 1983b). Briefly, rabbit antiserum raised against a papain-solubilized TMT081 microsomal fraction was extensively absorbed with normal rat tissues until no precipitin line could be detected in immunodiffusion against normal rat tissues or serum. The absorbed antiserum was then mixed with normal rat serum (10:1) and used as a reagent for the detection and quantification of mammary tumor, TMT081-associated antigen (MTA). A specific amount of ^{125}I-labeled MTA was incubated with a standardized amount of anti-MTA reagent (No. 7146) in the presence of varying amounts of test solutions. One hour incubation at 37° C was followed by treatment with goat anti-rabbit antiserum. Washed precipitate was counted for radioactivity. One unit of antigen is defined as the amount that will cause a 50% inhibition in the amount of radioactivity precipitated.

RESULTS

Expression of marker antigen MTA in rat mammary tumors grown in nude mice. The cell surface antigen, MTA, primarily associated with the metastatic rat mammary tumor, TMT081, is present in lactating rat mammary tissues as well as in nonmetastatic carcinoma cells. However, different tumors express different amounts of this antigen. To determine whether the rat mammary tumors grown in nude mice were still capable of expressing MTA, the levels of the antigen in TMT081 and MT100 tumors from rats as well as nude mice were measured. The results (Table 1) show that the metastatic tumor lines TMT081 and TMT081-MS, which did not grow lethally in nude mice, retained their phenotype as defined by the occurrence and shedding of MTA. In the case of nonmetastatic MT100 and the cell line MT100-TC, the antigen MTA was detectable only on the tumor both in rats and nude mice and

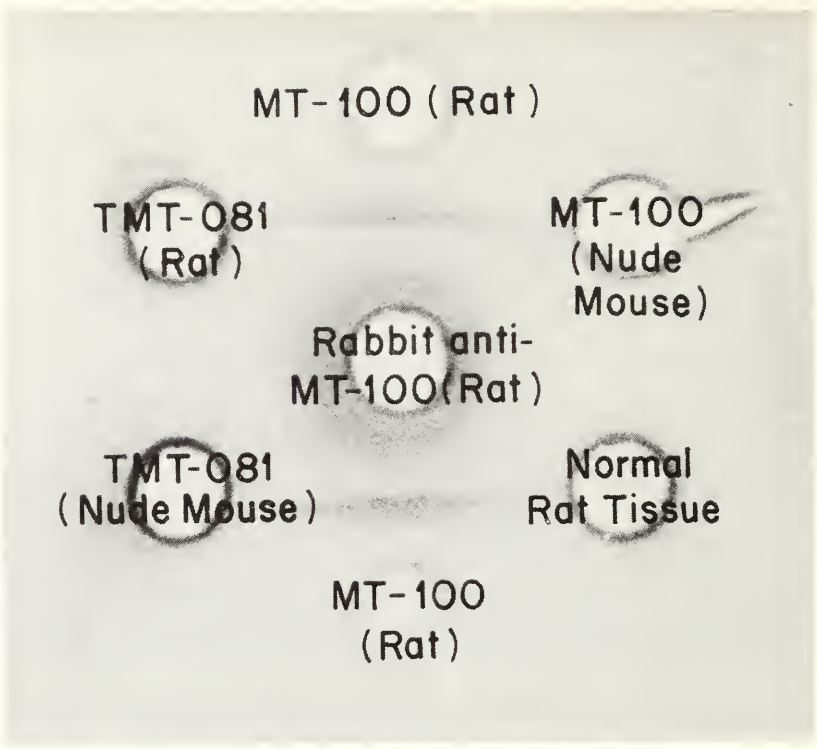
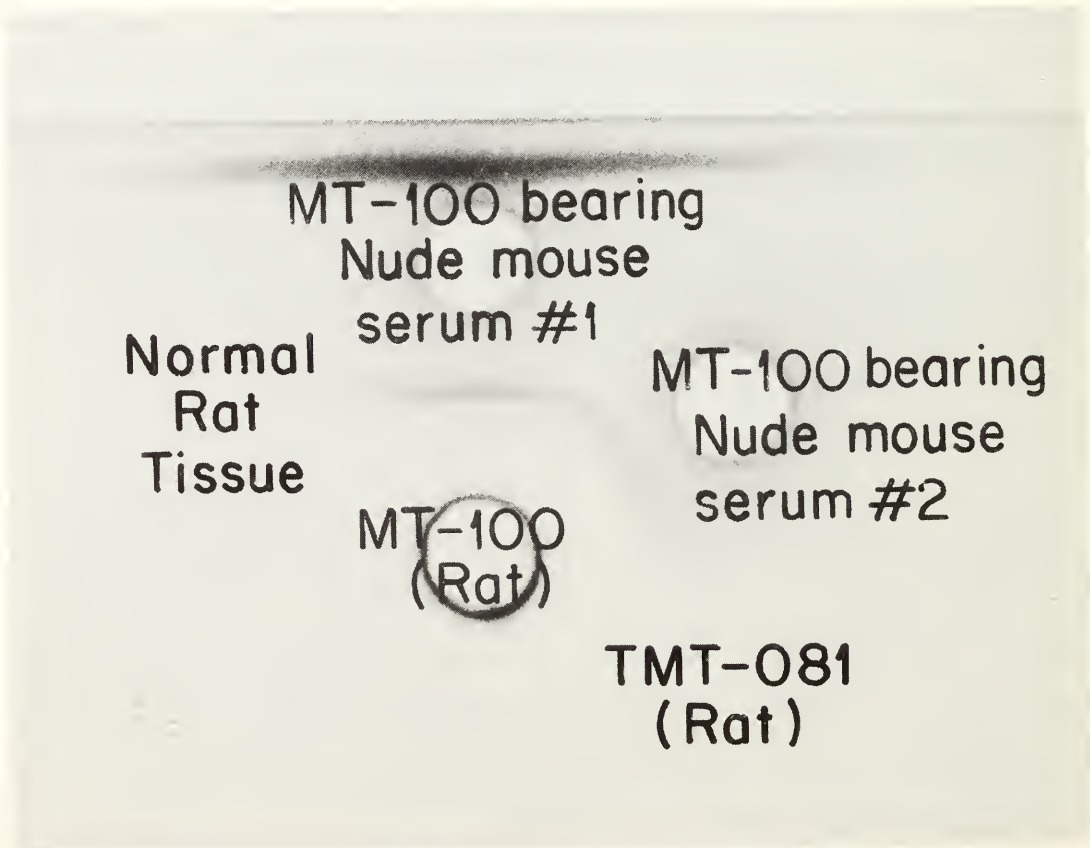


Figure 3. Immunodiffusion experiment demonstrating that MT100 antigens not shared by TMT081 were recognized by the specific rabbit antisera.



Fiqure 4. Immunodiffusion experiment demonstrating the presence of tumor-specific antibody in the sera of nude mice bearing progressively growing rat mammary tumor, MT100.

was never shed in detectable amounts (Ghosh, *et al.,* 1983b). It is apparent from Table 1 that the original rat mammary tumor MT100 did not lose its ability to express this MTA when transplanted into nude mice.

Antigenicity of rat tumors. In order to determine the phenotypic changes that occur in these rat tumors during passage in nude mice, sera and tumors from different groups of nude mice were tested against rabbit anti-TMT081 and rabbit anti-MT100 reagents by double immunodiffusion. Thus, in these experiments, rabbits were used as the indicators of phenotypic changes in the rat tumors as they grew in different hosts. As shown in Figure 1, absorbed rabbit anti-MT100 (rat) recognized more than one antigen in the homogenate of MT100 (rat) tumors. On the other hand, rabbit antisera produced against nude mice-derived MT100 yielded a single precipitin line that showed identity with one of the two precipitin lines observed using rabbit anti-MT100 (rat). Thus, MT100 from nude mice did not have detectable levels of the two antigens exhibited by the same tumor obtained from rats. To confirm this, the absorbed rabbit anti-MT100 (rat) and rabbit anti-MT100 (nude mice) were tested against the homogenate of MT100 (nude mouse) by immunodiffusion. The results (Figure 2) clearly indicate that both anti-MT100 (rat) and anti-MT100 (nude mouse) detect a single and identical antigen from the nude mouse-derived MT100.

To determine whether the antigenic determinants as revealed in Figures 1 and 2 were tumor-associated, homogenates of normal rat tissues as well as those of tumors and nonmammary tumor PW739 were tested for their reactivity with rabbit anti-MT100 reagents. Figures 2 and 3 show no discernible precipitin line against any other tumor or normal tissue tested except MT100 tumor homogenates.

In separate experiments, the presence of MT100 antigens in sera of rats and nude mice could not be demonstrated. This result indicates that the antigens recognized in MT100 tumor homogenates by specific rabbit antisera reagents were not shed in detectable amounts in the sera of tumor-bearing animals.

Immunodiffusion experiments were also performed using rabbit anti-TMT081 reagents against the tumor homogenates of TMT081 from both rats and nude mice. Single precipitin lines showing complete antigenic identity between TMT081 (rat) and TMT081 (nude mice) tumors were obtained. Furthermore, these precipitin lines also showed complete identity with those produced by MTA, the common TMT081-associated antigen characteristic of rat metastatic mammary tumor (Ghosh, *et al.,* 1978). Thus, TMT081 tumor, whether from rats or from nude mice, induced identical antibody response in rabbits.

Presence of circulating antibody in nude mice bearing MT100 tumors. When homogenates of MT100 tumors from rats were tested by immunodiffusion against the sera of MT100 tumor-bearing nude mice, the presence of circulating antibody was clearly detected (Figure 4). No such precipitin line, however, was observed when the same sera were tested against rat tissue preparation. Furthermore, no precipitin line was obtained against the sera of MT100 tumor-bearing mice, if the MT100 tumor preparation was pretreated or absorbed with the rabbit anti-MT100 (rat). However, pretreatment with the rabbit anti-MT100 (nude mouse) did not eliminate the precipitin line. Therefore, serum from MT100-bearing mice contains antibody to only the MT100 antigen, which is present in rats but not mice. The absence of one antigen in nude mice-derived MT100 with concomitant appearance of circulating antibody suggests that the latter prevents or blocks the expression of the reacting antigen, thus causing immunomodulation of tumor phenotype. Furthermore,

since no such antibody response occurred in rats, transplantation back into rats of nude mouse-derived MT100 allows reexpression of the blocked or unexpressed antigen.

The specific immune complex containing shed MTA could, however, be detected in TMT081-bearing rat sera (Ghosh and Roholt, 1984). Since TMT081, unlike MT100, sheds large amounts of tumor-associated antigen, MTA (Ghosh, *et al.,* 1978, 1979), no free antibody was, however, detectable in the circulation of tumor-bearing rats (Ghosh and Roholt, 1984). When homogenates of TMT081 tumor from rats were tested in the same way against the sera of MT100 and TMT081-bearing nude mice, no antibody could be detected by immunodiffusion. Thus, only the sera of MT100 tumor-bearing nude mice reacted specifically to MT100 but not TMT081 tumors.

CONCLUSIONS

This paper describes the changes in some of the phenotypic characteristics of rat mammary tumors during their progression in athymic nude mice as well as in syngeneic rats. The phenotypic profiles of these tumors as they manifest under different microenvironments in different hosts may be important in our understanding of tumorigenic and metastatic processes.

The results of the present study indicate that the properties of the metastatic and nonmetastatic rat adenocarcinoma cells exhibit considerably different biologic and antigenic properties when propagated in athymic nude mice. Whether the absence of a functional thymus in these hosts causes the reversal of the metastatic behaviors of these rat adenocarcinoma cells is not understood. Athymic nude mice are often used in propagating heterologous tumors, including those of human origin. In most cases, such heterologous tumors grow only at the site of transplantation but apparently maintain their biologic and histologic characteristics (Kim, *et al.,* 1982). However, in the case of rat mammary tumor MT100, hematogenous spread to different organs was observed (Kim, *et al.,* 1982). The spontaneously metastatic line, TMT081, grows in athymic nude mice only for a few days (Kim, *et al.,* 1982), but the nonmetastatic mammary tumor, TM100, grows and kills the nude mice hosts and at the same time undergoes a distinct but reversible change in its ability to adapt to this particular host. In this study, TMT081, the metastatic line, during the brief period of its growth in nude mice does not undergo any detectable phenotypic change as determined by the expression of its characteristic marker antigen, MTA. On the other hand, the nonmetastatic MT100 tumor, which expresses two cellular antigens during passage in WF rats, fails to express one of the two antigens as it grows in nude mice. Furthermore, concomitant with the lack of expression of the antigen, there is a discernible humoral response in the nude mice bearing MT100 tumors. In addition, the unexpressed antigen in the nude mice produced the antibody response in those mice. This response is considered to be the result of immunomodulation, because the production of serum antibody would have a blocking effect on the expression of the reacting antigen. This conclusion is supported by the fact that transplantation back into rats of the nude mouse-derived MT100 causes reexpression of this antigen, probably because there is no detectable antibody response in rats (Ghosh, *et al.,*

1983a). Interestingly, such changes are often accompanied by the acquisition of metastatic characteristics by MT100, which though invariably nonmetastatic in rats, localizes to the liver and lungs of nude mice besides growing at the site of implantation (Kim, *et al.,* 1982). However, these MT100 tumors from nude mice revert to nonmetastatic form after transplantation in the syngeneic hosts. The rat metastatic mammary tumor, TMT081, on the other hand, has been shown to induce humoral response in the syngeneic hosts (Ghosh and Roholt, 1984). Thus, it appears that the antigenicity of a tumor may be important in the expression of its metastatic phenotype.

Immunomodulation of tumor phenotype has been described before (Nicolson, 1984). However, the author is not aware of any previous studies addressing the relationship between pathogenicity and antigenicity of tumors. The experiments reported here have shown that the acquisition of a metastatic behavior could be influenced by the host's humoral immune response to the tumor. However, whether the lack of mature T cells in nude mice and the consequent lack of T-cell-derived factors contribute differently to the growth and progression of MT100 and TMT081 tumors is not understood at present and will be pursued in the future.

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