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Influence of Mammary Tumor Virus Infection on the Acceptance or Rejection of Transplanted ML+ Leukemias* (32611)

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The ML (Mammary Leukemia) antigenic system was first recognized during a study of cytotoxic antibodies produced by mice immunized against histoincompatible leukemias of DBA/2 mice (1,2). These antisera, after removal of isoantibodies by absorption *in vivo* in normal DBA/2 mice, were still cytotoxic for several DBA/2 leukemias. Absorption analysis of the sera *in vitro* indicated that the antigen identified in DBA/2 leukemias was present also in lactating mammary tissues and mammary tumors of mice infected with the mammary tumor virus (MTV), but was not demonstrable in any other tissues, normal or malignant, from either MTV+ or MTV— mice. On the basis of these findings it was concluded that this cellular antigen was derived from MTV. The designation ML (Mammary Leukemia) was chosen to indicate its presence in both mammary tumors and certain DBA/2 leukemias (designated ML+ leukemias).

Recently we have demonstrated by immunodiffusion a soluble antigen, MTV-s1, which like ML antigen is present both in mammary tissues infected with MTV and in certain leukemias of DBA/2 mice (3). This antigen is a subunit of MTV and is released by treatment of the intact virion with ether. It is not known whether these two antigens, ML cellular antigen and MTV-s1 soluble antigen, have the same specificity or different specificities; their occurrence is invariably linked, however, because they both are associated with infection by MTV.

This report is concerned with the question whether ML antigen in ML+ leukemias is capable of eliciting resistance to the growth of transplanted ML+ leukemias or of transplanted mammary tumors. For this purpose ML+ leukemias were inoculated into the following hosts, all genotypically histocompatible: (a) mice infected with MTV, (b) mice of strains freed of MTV by foster-nursing, and (c) reciprocal F_1 hybrids from various crosses between MTV+ and MTV— mice. Experiments in which resistance to mammary tumor transplants was successfully transferred by peritoneal cells from donors immunized with an ML+ leukemia confirmed that the specificity of this resistance was determined by MTV.

Materials and Methods. Mice. These were obtained from our own colonies. To simplify the description of mouse strains the abbreviations used are shown in Table I.

Preparation and inoculation of cell suspensions. Leukemia cells. Cell suspensions were prepared from leukemic spleens by mincing with curved scissors in Earle's Balanced Salt Solution (EBSS). The released cells were washed once and resuspended in fresh EBSS.

Mammary tumor cells. Suspensions of mammary tumor cells were prepared by slow circulation of tissue fragments in EBSS containing 250 mg trypsin 2× crystallized), 25 mg collagenase, and 2 mg deoxyribonuclease

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TABLE I. Abbreviated Designations of Mouse Strains.

Abbreviated designation	MTV infection	Full designation or description
BALB	—	BALB/c
C3H	+	C3H/An
C3Hf	—	C3Hf/Bi
DBA	+	DBA/2
BALB+^{C3H}	+	Two BALB sublines infected with MTV by foster-nursing on a C3H ♀ or a DBA ♀ (F_4–F_6 at the time of these experiments).
BALB+^{DBA}	+	
DBAf	—	DBA subline freed of MTV by caesarian section and foster-nursing on a C57BL ♀ (F_4–F_6 at the time of these experiments).

(Worthington Biochemical Corp., Freehold, N.J.) per 100 ml(4). The released cells were washed twice and resuspended in EBSS containing 5% fetal calf serum (previously heated for 30 min at 56°C) with penicillin and streptomycin.

Peritoneal cells. Suspensions of peritoneal cells were collected 3 days after the donors had received an ip injection of 1 ml of a 3% starch suspension. The cells were obtained by washing out the peritoneal cavities of living mice through a fine-bore polyethylene catheter with 4–5 ml EBSS containing 0.5 units of heparin, 100 units of penicillin and 0.1 mg of streptomycin per ml; the cells were washed once and resuspended in EBSS, this being carried out in the cold to prevent loss of cells from adherence to the glass (5).

The viability of all cell suspensions was determined by staining with trypan blue. Suspensions of leukemia cells and peritoneal cells contained less than 10% dead cells; suspensions of trypsinized mammary tumor cells contained less than 20% dead cells.

All inoculations of cells were subcutaneous in a previously shaved dorsal surface. In each experiment the control mice inoculated with leukemia cells or mammary tumor cells alone were divided into two groups. The first control group was inoculated *before* all experimental groups and the second control group *after* all experimental groups. Cell counts were adjusted to give the required number of cells in an injection volume of 0.2 ml/mouse. Tumor size is recorded as the average of two diameters at right angles to one another.

Results. Growth of ML+ and ML— leukemias in MTV+ and MTV— mice. The ML+ leukemia *DBA/2 ♂ SL2* (see Reg. 1) was inoculated into a variety of MTV+ and MTV— mice. Table II shows that all mice infected with MTV [DBA, (DBA × BALB) F_1, (C3H × DBA)F_1, (BALB+^{C3H} × DBA)F_1, and (BALB+^{DBA} × DBA)F_1] were susceptible to the leukemia and died of progressive disease. In contrast, MTV— mice were considerably resistant to transplants of *DBA/2 ♂ SL2;* rejection occurred in 4/6 (BALB × DBA)F_1, 9/15 (C3Hf × DBA)F_1, and 14/24 DBAf mice. Resistance was demonstrable in both males and females.

The specificity of the rejection of ML+ leukemias in MTV— mice is shown by the fact that MTV— mice did not exhibit resistance to transplants of histocompatible ML— leukemias. Table II shows that the three ML— leukemias tested grew progressively in both MTV— and MTV+ mice with no significant difference in survival times.

Another ML+ leukemia, *DBA/2♀SL1,* grew equally well in MTV— and MTV+ mice (Table III), but this leukemia grows very rapidly and must be transplanted every 6–7 days. With inocula of similar cell numbers *DBA/2 ♂ SL2* is transplanted every 10–12 days and the cells are more sensitive to cytotoxic ML antisera than are the cells of *DBA/2♀SL1* (see Ref. 1). It was therefore considered that the lack of resistance of MTV— mice to *DBA/2♀SL1* might be a result of inadequate antigenic stimulation. To test this possibility MTV— mice were preimmunized with *DBA/2 ♂ SL2* and then

TABLE II. The Growth of ML+ and ML— Leukemias in MTV+ and MTV— Mice.

Inoculum (sc)		Recipients			No. of mice	No. of mice surviving on day—			
Leukemia[a]	No. of cells	Genotype[b]	Sex	MTV		30	60	90	120
DBA/2 ♂ SL2 (ML+)	1×10^5	DBA	♂	+	6	2	0		
		DBAf	♂	—	5	3	3	3	3
		(BALB × DBA)F_1	♂	—	6	6	4	4	4
		(DBA × BALB)F_1	♂	+	6	1	0		
		(BALB+^{DBA} × DBA)F_1	♂	+	6	2	0		
		(BALB+^{C3H} × DBA)F_1	♂	+	6	2	0		
DBA/2 ♂ SL2 (ML+)	2×10^5	DBA	♂	+	4	2	0		
		DBA	♀	+	5	1	0		
		DBAf	♂	—	8	8	6	6	6
		DBAf	♀	—	11	11	5	5	5
DBA/2 ♂ SL2 (ML+)	2×10^5	(C3H × DBA)F_1	♂	+	15	5	1	0	
		(C3H × DBA)F_1	♀	+	14	6	2	0	
		(C3Hf × DBA)F_1	♂	—	8	8	5	5	5
		(C3Hf × DBA)F_1	♀	—	7	7	5	4	4
		(BALB+^{C3H} × DBA)F_1	♂	+	13	11	1	1	0
		(BALB+^{C3H} × DBA)F_1	♀	+	8	5	1	0	
DBA/2 ♂ DMBA4 (ML—)	1.3×10^5	(BALB × DBA)F_1	♂	—	5	0			
		(DBA × BALB)F_1	♂	+	5	0			
		(BALB+^{DBA} × DBA)F_1	♂	+	5	0			
BALB/c ♂ Estradiol 1 (ML—)	1.7×10^5	(BALB × DBA)F_1	♀	—	5	0			
		(DBA × BALB)F_1	♀	+	5	0			
		(BALB+^{DBA} × DBA)F_1	♀	+	6	0			
BALB/c ♂ SL9000 (ML—)	3×10^5	(BALB × DBA)F_1	♂	—	5	5	0		
		(DBA × BALB)F_1	♂	+	5	1	0		

[a] The abbreviations used in designating leukemias are: SL, spontaneous leukemia; DMBA, induced by 9,10-dimethyl-1,2-benzanthracene.

[b] The abbreviations used in designating mouse strains are given in Table I.

challenged two weeks later with *DBA/2♀SL1*. Table III shows that after preimmunization with *DBA/2 ♂ SL2*, MTV— mice were now capable of rejecting transplants of *DBA/2♀SL1*. The specificity of this preimmunization was confirmed by the observation that the ML— leukemia *DBA/2 ♂ DMBA4* grew progressively both in unimmunized MTV— mice and in MTV— mice that had been preimmunized with *DBA/2 ♂ SL2*.

Hyperimmunization of MTV— mice by progressively increased inocula of ML+ leukemia cells. In some experiments, MTV— mice that had rejected the initial transplant of the ML+ *DBA/2 ♂ SL2* leukemia were subsequently rechallenged with progressively increased inocula of this same leukemia. The (BALB × DBA)F_1 mice and DBA/2f mice eventually rejected intraperitoneal transplants exceeding 300×10^6 *DBA/2 ♂ SL2* cells. Immunization of (C3Hf × DBA)F_1 mice was discontinued after a final intraperitoneal challenge of 25×10^6 *DBA/2 ♂ SL2* cells.

Transfer of resistance to transplants of mammary tumors with peritoneal cells from mice immunized with an ML+ leukemia. These experiments were performed with the object of determining whether resistance to ML+ leukemic transplants would extend to transplants of mammary tumors. BALB mice hyperimmunized against the histoincompatible ML+ leukemia *DBA/2 ♂ SL2* or the histoincompatibe ML— leukemia *AKR♀K36* (control: G+ leukemia, see Ref. 6) were used for this purpose. The mammary tumors were obtained from the

TABLE III. Induction of Resistance to Transplants of a Weakly Antigenic ML+ Leukemia by Previous Inoculation of a Strongly Antigenic ML+ Leukemia.

Inoculum (sc)		Recipients				No. of mice surviving on day—			
DBA/2 leukemia	No. of cells	Genotype[b]	Sex	MTV	No. of mice	10	20	30	40
♀ *SL1* (ML+)	1×10^5	(BALB × DBA)F_1	♂	—	5	5	0		
		(DBA × BALB)F_1	♂	+	4	4	1	0	
		(BALB+^{DBA} × DBA)F_1	♂	+	5	5	2	0	
♀ *SL1* (ML+)	1×10^5	(BALB × DBA)F_1	♀	—	7	7	2	0	
		(BALB × DBA)F_1 pre-immunized with ML+ leukemia *DBA/2 ♂ SL2*[a]	♀	—	5	5	4	4	4
♂ *DMBA4* (ML—)	2×10^5	(BALB × DBA)F_1	♀	—	5	1	0		
		(BALB × DBA)F_1 pre-immunized with ML+ leukemia *DBA/2 ♂ SL2*[a]	♀	—	5	2	0		

[a] 7×10^6 *DBA/2 ♂ SL2* leukemia cells inoculated sc 14 days previously.

[b] The abbreviations used in designating mouse strains are given in Table I.

BALB+^{C3H} and BALB+^{DBA} sublines. The method used was the transfer of resistance by peritoneal cells obtained from living mice, rather than direct challenge, in order to preserve the immunized donors for further experiments (5). Peritoneal cells collected by catheterization 3 days after starch stimulation were mixed with suspensions of trypsinized mammary tumor cells. These cell mixtures were immediately injected subcutaneously into normal BALB female mice. As the peritoneal cells, the tumor cells and the recipients are all of BALB genotype, reactions based on isoantigenic disparity are excluded.

An inoculum of 1×10^5 BALB+^{C3H} mammary tumor cells was permanently suppressed by 30×10^5 admixed peritoneal cells from BALB mice immunized with the ML+ leukemia *DBA/2 ♂ SL2*. The outgrowth of 18×10^5 BALB+^{C3H} mammary tumor cells in BALB mice was considerably delayed by 54×10^6 peritoneal cells from the same source. In a third experiment, shown in Table IV, the outgrowth of 6.9×10^5 BALB+^{DBA} mammary tumors was markedly delayed by 13.8×10^6 immune peritoneal cells. Mammary tumor cells inoculated alone and mammary tumor cells mixed with peritoneal cells from BALB mice immunized against an ML— leukemia grew progressively and at similar rates.

Discussion. The ML antigenic system was defined serologically with cytotoxic antisera in

TABLE IV. Suppression of Inoculated BALB+^{DBA} Mammary Tumor in BALB/c ♀ Mice by Admixed Peritoneal Cells from BALB/c Mice Immunized with the ML+ Leukemia *DBA/2 ♂ SL2*.

Source of peritoneal cells[a]	Tumor diameter (mm) on day[b]			
	13	17	20	22
None	7.5	14.2	17.6	20.2
BALB/c ♀ mice immunized with ML— leukemia *AKR ♀ K36*	6.4	13.4	17.6	18.9
BALB/c ♀ mice immunized with ML+ leukemia *DBA/2 ♂ SL2*	0.0	0.0	1.0	2.4

[a] Peritoneal cell suspension added to inoculum of 6.9×10^5 viable trypsinized mammary tumor cells in a ratio of 20:1.

[b] Average for 5 recipients in each of the 3 groups.

tests with DBA/2 leukemias and the presence of this cellular antigen was shown to be determined by MTV (1,2). Later studies with precipitating antisera against purified MTV showed that MTV-s1 antigen, a subunit of MTV, is present in ML+ leukemias but not in ML— leukemias (3). Thus two independent immunological approaches indicate the presence of MTV antigen in certain leukemias of DBA/2 mice, and the absence of MTV antigens from leukemias of other MTV+ strains.

The possession of common antigens by mammary tumors and ML+ leukemias indicated by the serological data is paralleled by the results of transplantation. Thus MTV— mice immunized with ML+ leukemias acquire resistance to mammary tumor transplants, evidenced by the capacity of immunologically competent cells from these mice to suppress transplants of mammary tumors.

Acceptance or rejection of transplanted ML+ leukemias is governed by whether or not the recipient mice are congenitally infected with MTV. Uninfected mice of the strain of origin can reject ML+ leukemias but not ML— leukemias. The reason that MTV+ mice cannot respond to transplants of ML+ leukemias is presumably immunological tolerance arising from congenital and life-long infection with MTV. Adult MTV— mice are not immunologically tolerant and so are capable of responding to such transplants. This is the explanation first put forward by Morton to account for his demonstration that MTV— mice are resistant to transplants of mammary tumors whereas MTV+ mice are not (7). Extensive experimental support of this concept has been provided by Lavrin, Weiss, and their colleagues (see 8).

The question as to why ML+ leukemias are restricted to the DBA/2 strain cannot be answered with certainty. Antigenic conversion (9) produced by secondary infection with MTV is not a satisfactory explanation for the presence of ML antigen in leukemia cells because it does not account for the absence of ML antigen from leukemias of MTV+ mouse strains other than DBA/2. Furthermore it is not established that MTV persists as a proliferative secondary infection in either normal or malignant cells other than those of mammary origin. In this respect it apparently differs from the murine leukemia viruses, which infect a wide variety of cells under both natural and experimental conditions and impart to them the cellular antigens characteristic of these viruses (2,10,11). A more acceptable alternative explanation is that the DBA/2 strain carries a variant virus that is not found in other mouse strains. This might be, for example, (a) a mutant MTV with leukemogenic properties, (b) a mutant MTV with the property of replicating in leukemia cells, as MTV usually does not, (c) a recombinant between MTV and a leukemia virus, or (d) a defective leukemia virus requiring MTV for its maturation. The occurrence of DBA/2 leukemias with the G (Gross) cellular antigen (6) indicates that infection with wild-type Gross virus does occur in DBA/2 mice, providing the opportunity for genetic interaction between MTV and leukemia virus. Some ML+ leukemias have cellular antigens associated with wild-type murine leukemia virus but others do not, so this sheds no light on the virus determining ML antigen. In this respect ML+ leukemias do not differ from the leukemias of probably all strains, which also may or may not be G+.

Thus although there is a good case for regarding the DBA/2 virus inducing ML antigen in leukemias as unique, its relation to the murine leukemia viruses is still uncertain.

Summary. ML (Mammary Leukemia) cellular antigen, defined by specific cytotoxic antisera, is present on the cells of mammary tumors of MTV-infected mice and on the cells of some DBA/2 leukemias (ML+ leukemias). Transplants of ML+ leukemias are rejected by genotypically histocompatible mice not infected with MTV, but not by MTV-infected mice of the same genotype. Heightened resistance to transplants of mammary tumors can be transferred to recipient mice by immunologically competent cells from donors that have rejected ML+ transplanted leukemias.

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Anaerobic Rat Heart: Effect of Glucose and Krebs Cycle Metabolites on Rate of Beating* (32612)

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Energy production in mammalian hearts exposed to conditions of anaerobiosis has been considered insufficient to maintain beating (1–5). This phenomenon has also been held to be common to other endergonic functions occurring in a variety of mammalian cells (6). However, it has since been shown in an investigation of one such case, active cation transport in anaerobic rat liver slices, that electron accepting metabolites could maintain activity at a level comparable to that attained in oxygenated tissues (7,8). The rationale behind these studies was that electron accepting metabolites should theoretically enhance anaerobic energy production by stimulating (A) mitochondrial phosphorylation and (B) regeneration of cytoplasmic NAD. However, the ability to stimulate anaerobic energy production might be peculiar to liver since many of its cells appear to have a low requirement for oxygen. The intent of the present investigation was to determine if a comparable anaerobic stimulation of cellular function could be achieved in a very active tissue having a high requirement for oxygen. Heart was selected due to its overriding importance in the maintenance of life processes. In addition the automaticity of myocardial contractions provides a physiological parameter which could be easily observed under controlled conditions *in vitro*. Heart perfusions were conducted under an oxygen atmosphere followed by a period of anaerobiosis with media designed to stimulate glycolytic and mitochondrial ATP production. Myocardial contractions under anaerobiosis required the presence of glucose; rate of beating was concentration dependent. Anaerobic myocardial rate was significantly elevated by the further addition of mitochondrial metabolites.

The perfusion procedure was essentially that of Morgan *et al.* (4). Hearts were from male, Sprague-Dawley rats (300–350 gm). A pulsatile pump maintained coronary flow at 7 ml/min while the pressure was allowed to vary. Electrodes were implanted in the walls of the right and left ventricle and connected to an Offner dynograph recorder. Beating was found to be regular in almost all preparations. Arrhythmias occurred infrequently and appeared to bear no relationship to any specific experimental protocol. Rates of ventricular contractions obtained by direct visual observation were identical to those obtained by counting recorded R waves. Heart rates were

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