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Macrophage Migration Inhibition and Lymphocyte Stimulation with Mammary Tumor Virus Associated Antigens in Balb/c Mice (39179)

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We have previously reported that MTV-negative mouse mammary tumors which were originated by carcinogenic treatment with 7, 12 dimethylbenz(a)anthracene (DMBA) could be either immunogenic or nonimmunogenic to their host as measured by lymphocyte transformation reactions (1). Extracts of these tumors have failed to elicit reactions in lymphocytes from normal MTV-negative Balb/c mice. In more recent studies in our laboratories, it was observed that regardless of their immunogenic potential, these tumors failed to exert cross-reactive lymphocyte responses or stimulation of lymphocytes from MTV-positive Balb/cfC$_3$H mice either normal or implanted with MTV-positive mammary tumor (2). It was inferred from these findings that the D1-DMBA tumors maintained in Balb/c mice are devoid of MTV antigens. Similar conclusions were reached by Blair *et al.* (3) based on measurements of immune cytotoxic reactions. Moreover, radioimmunoassays have further confirmed the absence of MTV in these tumors (Parks, personal communication). All these findings are in accord with the previous reports that tissues of young Balb/c mice and the mammary tumors that occur occasionally in this line (Balb/cCrgl) are devoid of MTV (3–5). Against this background came the unexpected findings that the lymphocytes of Balb/c mice were reactive to MTV. Based on measurements of lymphocyte stimulation and inhibition of migration of macrophages, we have shown sensitivity of lymphocytes from MTV-negative Balb/c mice to VA but not to tumor specific antigens. These findings and their implications are presented in this report.

Materials and methods. Mice. Balb/c mice (MTV-negative) and Balb/cfC$_3$H (MTV-positive) were obtained initially from the Cancer Research Laboratories of the University of California. Geographically separated colonies of these mice are being maintained in our laboratories. The initial introduction of virus into the mice was achieved at the University of California by foster nursing the MTV-negative Balb/c on MTV-positive C$_3$H mice. Virgin mice ranging in age from 8 weeks to 3 months were used in these studies.

Tumors. D1-DMBA-3 was kindly provided by Dr. Phyllis Blair. Spontaneous mammary tumors arising in Balb/cfC$_3$H were transplanted in the subline of origin. No cross-reactions have been observed between these two types of tumors, further attesting to the absence of MTV in the D1-tumor lines.

Milks and MTV preparations. MTV-positive milk from RIII mice, milk from NIH Swiss mice, and purified MTV were kindly provided by the Virus Cancer Program from stocks supplied by the Meloy Laboratory.

Lymphocyte stimulation. The response of spleen lymphocytes to viral antigens and to the mitogens phytohemagglutinin-P (PHA, Difco) and concanavalin A (Con A, Calbiochem) was performed according to the previously published procedure (1).

Migration inhibition. Peritoneal exudate cells (PEC) were induced by intraperitoneal injections of 2 ml of 1% oyster glycogen, 48 and 24 hr prior to harvest, according to the method of Coggin *et al.* (6). PEC migration was assayed by an adaptation of the method of Pomales and Ortiz (7). In brief, milk or mitogen in appropriate dilutions was incorporated in 0.5% agarose dissolved in RPMI #1640 containing 20 μg/ml of heparin and centrifuged at 400g for 10 min. After two washings with the same medium, the cells were aspirated into gas-sterilized Intramedic polyethylene tubes (formulation PHF Adams PE-50) with the aid of a Corn-

wall syringe. The tubes were inserted into hematocrit needles for centrifugation in order to obtain a cell pack. Pieces of tubing containing the PEC were cut sterily and placed on the surface of the agar contained in Falcon (35 × 10 mm) dishes. Incubation was at 37° in a 5% CO_2 atmosphere for periods of 24 hr. Migration areas were traced by means of a camera lucida microscope attachment and quantified by use of a planimeter. Migration inhibition was calculated from the formula,

$$\% \text{ of migration inhibition} = \left[1 - \frac{AT}{AC}\right] \times 100,$$

where AT is area of migration in the presence of test substance and AC is area in control plates. Thirty percent inhibition of migration was taken as the lower limit of significance.

Results. Migration inhibition of peritoneal exudate cells. MTV-positive milk from RIII mice in a dilution range of 1:200 to 1:1600 caused significant inhibition of migration of PEC both from normal Balb/c and Balb/c mice bearing MTV-negative D1-DMBA tumors. Table I shows the mean levels of effect for each dilution of milk. Milk of NIH Swiss mice did not inhibit migration. Strikingly different results were obtained with PEC from Balb/cfC_3H mice. RIII milk did not cause significant inhibition of migration. Moreover, transplantation of the chemically induced MTV-negative D1-DMBA-3 tumors did not alter the degree of responsiveness in PEC of Balb/c mice and did not evoke a sensitivity to MTV in Balb/cfC_3H mice. However, positive reactions were obtained with PEC from mice of this subline after implantation with MTV-positive tumors derived from Balb/cfC_3H mammary carcinomas.

Lymphocyte stimulation of spleen cells. Table II shows a representative experiment on lymphocyte transformation using Balb/c animals stimulated by RIII milk, NIH Swiss milk, and purified MTV. It can be seen that definite increases in thymidine uptake were obtained in spleen cells of normal and tumor-bearing animals in the presence of purified virus or MTV-positive RIII milk. Milk from NIH Swiss mice gave negative or minimal stimulation. MTV-positive milk is a more readily available commodity than is purified MTV. When used in migration inhibition experiments as shown in Table I, such milk produced clear-cut results. This was not always the case, however, in blastogenic transformation reactions. In many instances, as shown in Table II, there was a prozone effect where low dilutions of milk caused little or no response in normal lymphocytes while higher dilutions evoked significant responses. Furthermore, in several experiments not presented here, the serial dilutions of milk produced biphasic responses. Finally, the antigens present in the milk often caused a higher stimulation in lymphocytes from mice bearing D1-DMBA-3 tumors as compared with lymphocytes of normal Balb/c mice. The reasons

TABLE I. INHIBITION OF PEC MIGRATION BY MOUSE MILK.[a]

Milk final dilutions	Balb/cCrgl		Balb/cfC_3H		
	No tumor	D1-DMBA-3 tumor	No tumor	D1-DMBA-3 tumor	Spontaneous mammary tumor
RIII					
1:50	0% (7)	5% (6)	18% (4)	0% (2)	10% (3)
1:200	43 (23)	38 (22)	10 (12)	8 (4)	35 (13)
1:400	35 (6)	27 (6)	14 (4)	19 (2)	36 (2)
1:800	36 (22)	40 (24)	4 (12)	11 (5)	51 (13)
1:1600	47 (4)	34 (4)	6 (2)	0 (3)	16 (2)
Swiss mouse					
1:200	11 (20)	16 (19)	9 (9)	5 (2)	8 (11
1:800	8 (20)	12 (18)	11 (9)	3 (2)	10 (11)

[a] Results expressed as a percentage inhibition of migration relative to controls containing no milk. In parentheses number of mice tested. Thirty percent inhibition is taken as the lower limit of significance.

TABLE II. [^{3}H]THYMIDINE UPTAKE BY SPLEEN LYMPHOCYTES OF NORMAL AND D1-DMBA-3 TUMOR BEARING BALB/cCRGL MICE IN THE PRESENCE OF MTV.[a]

	Normal	S.I.	D1-DMBA-3 tumor bearing	S.I.
GM	994 ± 99		1162 ± 107	
PHA (1:800)*	15389 ± 1361	(15.48)	17203 ± 998	(14.80)
Con A (1 μg)	123233 ± 9558	(123.37)	131168 ± 7203	(112.88)
RIII milk				
1:400	1509 ± 107	(1.51)	3209 ± 201	(2.76)
1:800	1965 ± 149	(1.97)	3456 ± 164	(2.97)
1:1600	3097 ± 248	(3.28)	4222 ± 556	(3.63)
1:3200	4192 ± 187	(4.44)	7203 ± 389	(6.19)
Swiss milk				
1:400	1127 ± 92	(1.13)	1104 ± 120	(0.95)
1:800	1002 ± 82	(1.01)	982 ± 71	(0.85)
1:1600	902 ± 71	(0.91)	1202 ± 127	(1.03)
1:3200	1073 ± 132	(1.08)	1009 ± 82	(0.87)
Purified virus				
1:200	2731 ± 298	(2.89)	3162 ± 287	(2.72)
1:1000	3822 ± 277	(3.85)	4222 ± 391	(3.63)

[a] Results expressed as cpm/10^6 cells. * Final dilutions. S.I. = stimulation index, defined as the ratio of cpm of stimulated cultures to cpm of unstimulated cultures.

for these findings are not known, but the apparent discrepancies could be manifestations of recognition of different antigens of viral or cellular origin in particulate or soluble form. In view of these considerations, most of the subsequent experiments were done using purified MTV.

Figure 1 summarizes the results of assays on 53 Balb/c mice whose lymphocytes were tested against two different dilutions of purified MTV. Positive responses were obtained with both dilutions (in some cases one dilution was more stimulatory than the other). There were no significant differences in the responsiveness of lymphocytes from normal and D1-DMBA-3 tumor-bearing mice. Most of the stimulation indexes clustered within a range of two to six.

As in the tests with PEC of Balb/cfC_3H where MTV caused no significant inhibition of migration, it also failed to induce positive responses in the spleen cells of these animals as measured by the lymphocyte transformation assay. The absence of response was first demonstrated by means of MTV-positive RIII milk (2). More recently it was confirmed, albeit in a small number of animals, using purified MTV. The results are given in Table III. It can be seen that none of the normal Balb/cfC_3H mice possessed reactive lymphocytes. In contrast, and again in parallel with migration inhibition tests,

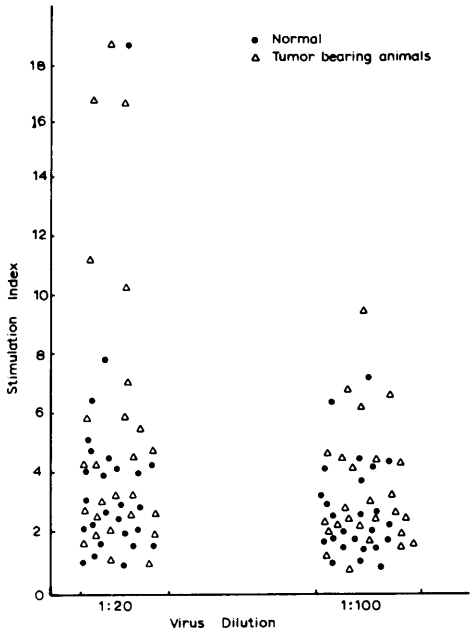

FIG. 1. Distribution of stimulation indexes of spleen lymphocytes in response to purified MTV, comparison of cells from normal and D1-DMBA-3 tumor bearing Balb/cCrgl mice. Stimulation index defined as the ratio of cpm of stimulated cultures to cpm of unstimulated cultures.

transplantation of spontaneous MTV-positive mammary tumors led to the appearance of lymphocytes reactive to MTV.

Discussion. It is generally accepted that

TABLE III. ABSENCE OF BLASTOGENIC RESPONSES OF NORMAL BALB/cfC$_3$H MICE TO PURIFIED MTV AND APPEARANCE OF THIS RESPONSE FOLLOWING IMPLANTATION WITH MTV POSITIVE TUMOR.[a]

Medium containing	Normal mice 1	2	3	4	5	6
GM alone	4041 ± 373	1118 ± 98	2773 ± 322	1167 ± 115	916 ± 63	
MTV						
1:200	N.D.	1219 ± 102 (1.09)	1112 (0.40)	995 ± 73 (0.85)	N.D.	
1:500	1695 ± 209 (0.42)	N.D.	N.D.	N.D.	1084 ± 75 (1.18)	
1:1000	N.D.	1152 ± 125 (1.03)	2574 ± 198 (0.93)	970 ± 107 (0.83)	N.D.	
1:2000	4075 ± 304 (1.01)	N.D.	1869 ± 202 (0.67)	N.D.	1024 ± 104 (1.12)	
			Tumor bearing mice			
GM alone	1618 ± 43	1998 ± 201	3164 ± 294	505 ± 33	3145 ± 297	1036 ± 88
MTV						
1:200	N.D.	4648 ± 433 (2.33)	92736 ± 8013 (29.31)	N.D.	1726 ± 787 (0.55)	3918 ± 403 (3.78)
1:500	7161 ± 636 (4.43)	N.D.	N.D.	940 ± 88 (1.86)	N.D.	N.D.
1:1000	N.D.	6536 ± 573 (3.28)	12280 ± 967 (3.88)	N.D.	12142 ± 1132 (3.86)	2783 ± 303 (2.69)
1:2000	4455 ± 322 (2.75)	N.D.	8138 ± 644 (2.57)	1021 ± 93 (2.02)	31424 ± 2973 (9.99)	N.D.

[a] N.D. = not done. Results expressed in cpm/10^6 cells. Number in parentheses represents stimulation index (S.I.), defined as the ratio of cpm of stimulated cultures to cpm of unstimulated cultures.

young Balb/c mice are free of MTV. According to published reviews, Balb/c mice are characterized by a low incidence of mammary tumors (8, 9). However, sublines of Balb/c mice maintained in some laboratories have been reported to have a heightened incidence of such tumors with increasing age, and other conditions of frequent matings (10). But even these tumors are apparently free of MTV or contain low levels of virus (11). Yet Balb/c mice possess cells that react to an antigen (or antigens) of MTV as determined in blastogenic and migration inhibition tests. Blair *et al.* (3) have reported that lymphocytes from Balb/c mice displayed a high level of cytotoxicity against cells from MTV containing spontaneous tumors arising in Balb/cfC$_3$H mice. These lymphocytes were not cytotoxic to the chemically induced D1-DMBA-3 or D1-DMBA-4 tumors which are free of MTV. As stated earlier, our studies and the assays in Dr. Parks' laboratory have also detected no MTV antigens in transplanted tumors derived from mammary carcinoma induced by DMBA in Balb/c mice. Thus, it has now been shown on the basis of three different parameters of cell-mediated immunity (cytotoxicity, blastogenic transformation, and migration inhibition) that lymphocytes from MTV-negative mice are capable of recognizing MTV antigens.

In contrast with these findings was the absence of immunologic recognition or response to MTV antigen (or antigens) on the part of cells from Balb/cfC$_3$H mice which carry the virus by vertical transmission. The PEC of these mice were not significantly inhibited in their migration by MTV and their spleen cells did not respond with increased DNA synthesis when exposed to this virus, suggesting that these animals may have been tolerant to MTV antigens. However, when these animals were implanted with an MTV-positive tumor derived from a spontaneous mammary carcinoma of a Balb/cfC$_3$H mouse, their PEC became strongly inhibitable and their spleen cells became responsive to MTV antigens. Two inferences can be made from these findings: either that tolerance was broken or that the response was due to an antigen other than that to which the animals were tolerant. MTV is known to contain

several different antigenic moieties (12–16) and, in fact, Blair *et al.* (17) demonstrated that the responses of Balb/c and Balb/cfC$_3$H cells were apparently directed to different antigens as determined by blocking of cytotoxic activity by Balb/c or Balb/cfC$_3$H sera.

The challenging question is: What is the origin of the reactive lymphocytes in the MTV-negative Balb/c mice which are characterized by a low incidence of mammary tumors? In the studies of Blair *et al.* (3), it was found that the development of responding lymphocytes was delayed until the thirteenth week of life. From this it was inferred that horizontal infection may have taken place. This possibility was strengthened by the recent finding by the same authors that mice kept outside of the animal quarters remained free of responding lymphocytes, whereas litter mates transferred from isolation to cages containing Balb/cfC$_3$H mice developed sensitive lymphocytes (18). This discovery has many implications to the problems of dissemination and acquisition of oncogenic RNA viruses and therefore should receive considerable attention. One important question raised by the finding of horizontal transmission is: Why do Balb/c mice remain free of MTV for most of their life span?

An alternative explanation that can be offered is that the reactivity of the lymphocytes is the result of sensitization to a viral protein of an endogenous origin. Several investigators have presented data compatible with activation of an endogenous MTV in Balb/c mice (5, 10, 19, 20). The probability of derepression of an MTV gene was substantiated by the molecular hybridization studies of Schlom *et al.* (21) which demonstrated the appearance of MTV-specific RNA in the cells of Balb/c as a result of aging and treatment with nonviral carcinogens. The genome is apparently repressed, and it is possible that genes coding for different constituents of the virus may be switched-on in a selective and sequential manner. Under such conditions there could be expression of individual viral antigens in the absence of production of infectious virus. There is now substantial evidence for the occurrence of viral structural proteins and glycoproteins in cells free of virus. This was first demonstrated by Hanafusa and collaborators (22, 23) in chicken embryo cells devoid of viral particles. More recently it was determined that the cell surface antigen, G_{ix}, which was originally thought to be a differentiation antigen of lymphoid cells (24) is also present in the envelope of murine leukemia virus (25, 26).

The two postulated causes of lymphocyte sensitization are not necessarily mutually exclusive and, in fact, provide new approaches to studies of the role of chromosomal and exogenous transmission of virus in the etiology of mammary tumors and in the emergence and functions of immunologic interactants and interplays that may affect the processes of infection, transformation, and tumor evolutions.

Summary. Lymphocytes of MTV-negative Balb/c mice are sensitive to one or more antigens of MTV as determined by blastogenic transformation and migration inhibition assays. The lymphocytes from MTV-positive Balb/cfC$_3$H mice are nonresponsive in these assays but become responsive after the mice have been implanted with MTV-positive tumors. The latter findings imply that there may be either a state of tolerance to MTV antigens which is broken by the tumor transplant or that the responses are directed towards antigens to which the host is not tolerant. The cause of the sensitivity of lymphocytes from MTV-negative Balb/c mice to MTV antigens has not been determined but two possible explanations are discussed: horizontal transmission and derepression of genetic information for viral antigen.

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