

Specificity and Age of Appearance of Natural Immunity to MTV Antigens (40131)

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Balb/c mice are characterized by a low incidence of spontaneous mammary tumors and by a low frequency of production of infectious mammary tumor virus (MTV). Not only are tissues of young Balb/c mice free of mammary tumor virus but breast carcinomas induced in Balb/cCrgl by 7,12 dimethylbenzanthracene do not contain antigens of MTV indicating the absence of virus expression (1). Radioimmunoassays for MTV p14 internal protein have yielded negative results and a preliminary survey for MTV antibodies directed against labelled virions (Fine, personal communication) further substantiates these conclusions as sera from these mice were found to be free of anti-MTV by the radioimmune precipitation test (2). Previous reports by several investigators (3-5) also indicate that the tissues of young Balb/cCrgl mice and the mammary tumors that occur occasionally in this line lack overt expression of MTV. Schlom *et al.* did report the presence of MTV-RNA in such tumors (6, 7). All this notwithstanding, Balb/c lymphocytes including those of the Crgl lineage react to MTV-positive RIII milk and to preparations of MTV derived from RIII milk as measured by increased levels of thymidine uptake and migration inhibition (8). In contrast, the same lymphocytes fail to respond to the MTV-negative milk from NIH Swiss mice (8, 9).

This report presents findings concerning the age of appearance and further tests to determine the specificity of this natural immune reactivity to MTV antigen(s).

Materials and methods. Mice and tumors. MTV-negative Balb/cCrgl virgin mice, 14 to 16 weeks old were used for the specificity experiments. For the age studies animals of 2, 8 and 16 weeks were employed. A transplantable MTV-negative mammary tumor (D1-DMBA-3) induced by 7,12 dimethylbenzanthracene was used at passage levels 8 to 10.

This tumor contains no mammary tumor virions under electronmicroscopic investigation, and contains no significant amounts of gp52 or p14 proteins in radioimmunoassays.

Viruses and antisera. MTV purified from RIII milk, Rauscher leukemia virus (RLV) as well as rabbit anti-MTV and goat anti-RLV were kindly provided by the Virus Cancer Program from stocks supplied by the Meloy Laboratories and the Frederick Cancer Research Center. In addition to these antisera, normal goat and normal rabbit sera (GIBCO, NY) were used as controls. All sera were heated at 56° for 1 hr prior to use, in order to inactivate complement components. MTV was purified at the Meloy Laboratories by a combination of rate zonal and isopycnic centrifugation and the stock used in our studies was 1 mg/ml of virus.

Lymphocyte transformation assays. The response of the splenocytes to the antigenic preparations was performed according to the previously published procedure (8). In addition, two sets of control cultures were included: baseline cultures containing lymphocytes in growth medium (GM) without any additive, and positive control cultures with the nonspecific mitogen concanavalin A (Con A) to attest to the general immunocompetence of the lymphocyte preparations.

Migration inhibition assays. Inhibition of migration of peritoneal exudate cells (PEC) was assessed by an adaptation (8) of the method of Pomales and Ortiz (10). Thirty percent inhibition of migration was considered as the lower limit of significance.

Radioimmunoassays. The previously published method of iodination (11) of viral antigens was employed. Determinations of MTV p14 antigen were obtained by the use of the competition radioimmunoassay described by Noon *et al.* (12).

Results. Lymphocyte transformation assays

were performed with splenocytes from normal and D1-DMBA-3 tumor bearing Balb/cCrgl mice. Figure 1 represents a composite of six separate experiments with four animals each. Responses to purified MTV were obtained in normal and tumor bearing mice. These responses were abolished when the virus was first incubated with anti-MTV for 1 hr at 37° prior to inoculation into tissue culture. The inhibitory activity was not due to toxic effect of anti-MTV serum in the cultures as determined with viability tests. Additional controls not shown in the figure included incubation of MTV with normal serum. This treatment had no effect on the responses to viral antigens.

Because MTV preparations may be contaminated with C type particles which could contribute to the responses, it was necessary to demonstrate that antibody against a C type oncornavirus would not alter this reaction. Lymphocytes from Balb/c normal mice and animals bearing tumors were tested with MTV incubated with goat anti-Rascher leukemia virus. In Fig. 2 it can be seen that there were no differences between these cultures and those containing only MTV. Moreover, we are unable to detect positive responses to

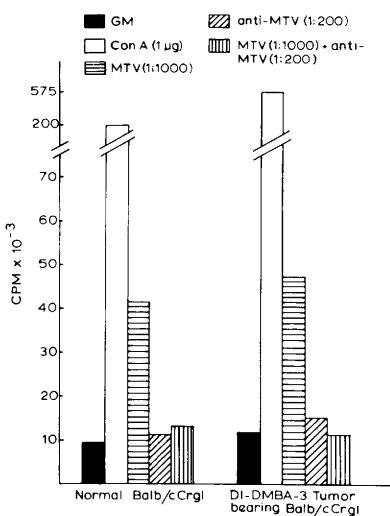


FIG. 1. Inhibition of blastogenic transformation by purified MTV after incubation at 37° with anti-MTV for 1 hr. Results expressed in cpm/10⁶ cells per cultures. Standard deviations never exceeded 10 percent. GM represents cultures where growth medium was added in place of stimulant. The MTV dilution of 1:1000 is equivalent to 1 µg MTV per culture.

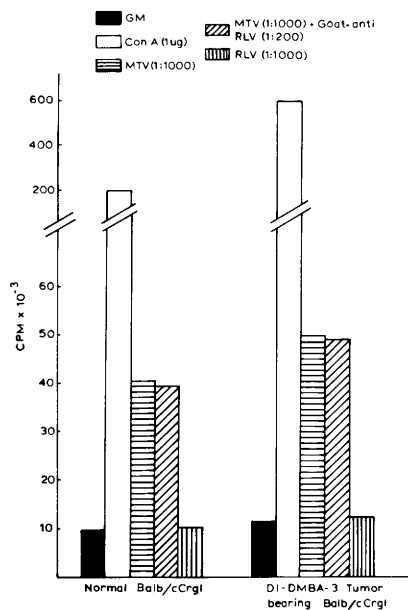


FIG. 2. Specificity of the reaction induced by MTV on lymphocytes from Balb/cCrgl as indicated by the lack of inhibitory effect of antiserum raised against a type C particle. Results expressed in cpm/10⁶ cells per culture. GM stands for controls containing only growth medium and lymphocytes.

RLV although in a few cases stimulation indexes of 1.3–1.5 were observed, (below level of significance).

We have previously described in another viral system the inhibition of PHA responses by antigen–antibody complexes (13). In order to rule out such an effect in the present system, RLV was combined with anti-RLV and added to the cultures prior to stimulation with MTV. As evidenced in Fig. 3 this combination did not alter either the background counts or the response to MTV.

To determine at what age the Balb/c mice develop the sensitivity towards the MTV antigen(s), studies were performed using lymphocytes from 2, 8 and 16 week old animals. As shown by the lymphocyte transformation assay presented in Fig. 4, we were able to detect low responses as early as 2 weeks of age in some animals. These responses were clustered around a range of stimulation indexes of 1.5–2.5. Stimulation index of two is generally considered as the lower level of significance. By 8 weeks of age, responses were positive in 90% of the cases. It is interesting that the responses were more notable

with lower dilutions of virus. At 16 weeks all animals were responsive to at least one dilution of the purified MTV.

Migration inhibition studies were performed using cells from animals 8 to 16 weeks of age. Two week old mice were not tested

due to the great difficulty of inducing PEC in such small animals. In Fig. 5 it can be seen that the results of these studies are essentially consistent with those obtained with the blastogenesis studies. It should be mentioned that some animals were responsive in one test and negative in the other.

Radioimmune assays using p14 as the competing antigen were performed on pools of thymuses and spleens of the same animals. As shown in Table I no significant amount of this antigen could be demonstrated in any of the preparations of tissues from Balb/c mice age 2 to 16 weeks (The sensitivity of this assay is 2 ng/mg). In previous analyses D1-DMBA-3 tumors of Balb/c mice were also found to be almost devoid of p14 antigens. A positive control consisted of an MTV-positive mammary tumor derived from a Balb/cfC3H mouse. In contrast with the negative findings of the Balb/c tissues the Balb/cfC3H tumor contained 283 ng/mg of p14.

Discussion. The previously described response of Balb/c lymphocytes to RIII MTV antigen(s) was confirmed in the present studies using both blastogenic transformation and migration inhibition assays. The specificity of the lymphocyte response to MTV was measured by means of virus neutralization reactions employing anti-MTV and anti-RLV sera. The former caused inhibition of blastogenic response to MTV while the latter had no effect. Furthermore, RLV did not induce

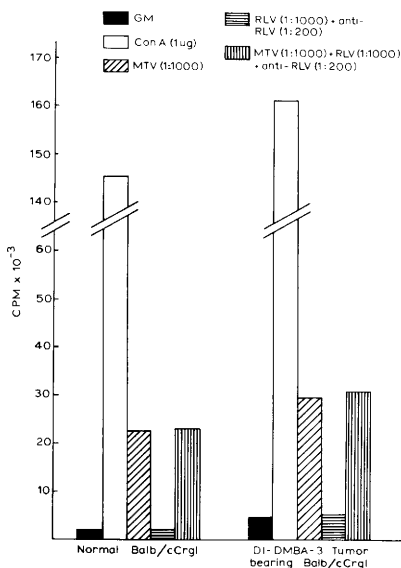


FIG. 3. Determination of the lack of inhibitory action of unrelated antigen-antibody complexes added to MTV used as stimulant in the lymphocyte transformation reaction. Results expressed in cpm/10⁶ cells per culture. Standard deviations were less than 10%. GM represents control cultures of lymphocytes in growth medium without any other additive.

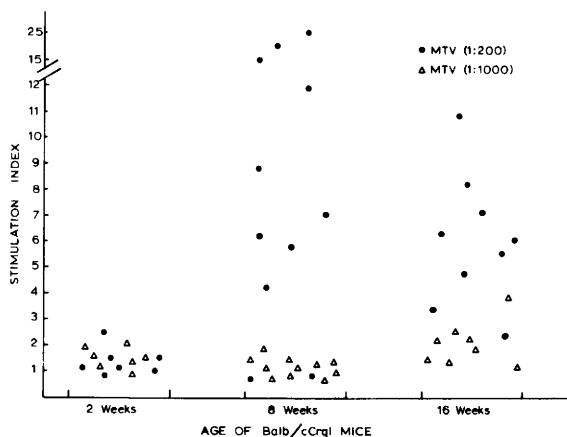


FIG. 4. Age of appearance of lymphocyte responsiveness towards mammary tumor viral antigens indicated by increased thymidine incorporation. Stimulation index is the value obtained dividing the cpm of cultures with MTV by the cpm of control cultures with no additive or in the presence of RLV. Each symbol represents the response from an individual mouse.

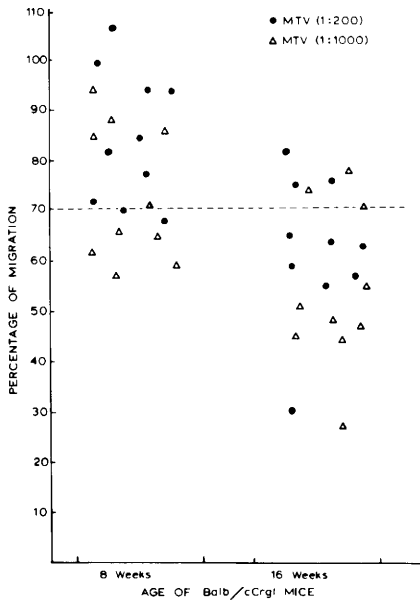


FIG. 5. Inhibition of migration of peritoneal exudate cells from young Balb/cCrgl mice in the presence of purified MTV. Migration indexes are calculated as previously described (8), in relation to control cultures where no MTV was added. Each symbol is the response of an individual mouse. Thirty percent inhibition is considered as the lower limit of significant responses.

TABLE I. ASSAY FOR MTV p14 IN THE TISSUES OF YOUNG Balb/cCrgl.^a

Strain	Age	Tissue	p14 concentration ng/mg tissue
Balb/c Crgl	2 weeks	Thymus	3
		Spleen	3
	8 weeks	Thymus	N.T.
		Spleen	3
16 weeks	Thymus	3	
	Spleen	3	
Balb/c fC3H		Spontaneous mammary tumor	283

^a Values obtained with the competition radioimmunoassay previously described (12).

transformation in Balb/c lymphocytes. In past work we found no positive responses with MTV-negative milk from NIH Swiss mice (8). These findings are interpreted as being consistent with the response being due to an RIII MTV associated antigen and not contamination with group-specific type C viral antigens.

Recently there have been many reports of

the existence of a natural cell-mediated immune reactivity against tumor associated or oncogenic virus-associated antigens (14, 15). These immune reactions can be detected in non-immunized normal individuals of several species including man (16, 17).

A phenomenon analogous to the one described by us has been reported by Blair *et al.* utilizing a lymphocytotoxic reaction as a measure of immune responsiveness (18). They have interpreted their findings as being evoked by infection with horizontally transmitted MTV (19). This is an intriguing aspect of MTV and murine mammary tumors in view of the fact that transfer of virus is generally considered to be limited to either genetic and/or milk transmission.

We have entertained a different interpretation, namely, that lymphocyte sensitivity to MTV antigen(s) originates from occurrence of a viral antigen(s) produced by the expression of a part of the viral genome in the host chromosome.

Although there is agreement that Balb/c mice are very low expressors of infectious mature particles, and this expression occurs only late in life (after 18 months), their cells appear to contain MTV nucleic acid sequences. Varmus *et al.* (20) found a close correlation between tumor incidence and the amount of viral genetic information, for example, the lactating mammary tissue of the high tumor strains GR and C3H had respectively a 1000 and a 100 virus genome equivalents per cell, whereas the corresponding tissues of the low mammary tumor incidence strain, Balb/c, had only one copy of the viral genome. The molecular probes of Schlom *et al.* (6) demonstrated an association between amount of viral RNA and age of Balb/c mice (essentially negative findings in young mice and occasionally positive findings in old breeders). Similar partial expression of type C viral genes in RNA and protein have been reported in Balb/c mice (21).

We have not yet determined the nature of the antigen responsible for the lymphocyte sensitivity. Radioimmunoassays have largely ruled out the MTV p14 antigen. The insignificant amounts of this antigen in the organs and chemically induced tumors of the Balb/cCrgl strain is further evidence for the absence of infectious MTV in these mice.

This antigen was found in high concentrations in tumors of MTV-positive Balb/cfC3H. Presence of viral antigens in cells of low virus expressor Balb/c mice has previously been reported by Gillette *et al.* (22, 23).

The early appearance of reactivity (low stimulation indexes at 2 weeks of age), is consistent with our contention. The relative low stimulation indexes compared to those of older mice are probably reflection of immaturity of the immune system.

Summary. Previous studies demonstrated that despite freedom from MTV and mammary tumors early in life Balb/cCrgl mice possess lymphocytes sensitive to mouse mammary tumor antigen(s) within 2–3 weeks of life. This sensitivity was manifested in blastogenic transformation and migration inhibition reactions using RIII milk and purified MTV from RIII milk. These reactions were not elicited with the MTV-negative milk from NIH Swiss mice. Preincubation of MTV with anti-MTV antibody inhibited the blastogenic reaction. In contrast, antibody raised against a type C murine oncornavirus had no inhibitory effect and did not by itself induce blastogenic transformation. The nature of the virus-related antigen responsible for the lymphocyte sensitivity has not been ascertained, nor has its location in the animals' tissue been determined. However, radioimmune assays for p14 antigen in multiple tissues of Balb/c mice have yielded insignificant values suggesting either incomplete expression of the genome and/or different levels of the diverse mouse mammary tumor virus antigenic components.

MTV-induced lymphocyte transformation is observed at low levels and in a small percentage of Balb/cCrgl mice as early as two weeks after birth in the blastogenic assay. At 8 weeks of age the responses in the lymphocyte transformation and migration inhibition assays are intermediate in frequency and/or intensity; by 16 weeks essentially maximal responses are noted.

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