Virus-dependent Cytostatic Activity to Mammary Tumor Cells of Lymphocytes from Normal Mice

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SUMMARY

A cytostasis assay has been used to study the natural immunity of mice to murine mammary tumor virus (MTV). Spleen cells from adults of all strains tested were found to be cytostatic to a variety of MTV-positive mammary tumor cell lines. Newborn spleen cells were unreactive in the same cytostasis assay. The degree of reactivity to the target cells was greater in spleen cell preparations from low MTV expressors than from syngeneic, high MTV expressors. The cytostasis was specific, since MTV antigens prepared from gradient-purified whole MT virions significantly blocked the reaction. In addition, spleen cells were totally nonreactive to MTV-negative cell lines. Other types of lymphoid cells, such as lymph node cells as well as peritoneal macrophages, were highly cytostatic under similar conditions. Spleen cells from nude athymic donors were not cytostatic. Since depletion of splenic T-lymphocytes by use of anti-Il serum also did not significantly affect cytostasis, it was concluded that T-cells were required for initiation of immunity to MTV but that the effector cells were not Il positive. Blocking factors were found to exist in the sera of mammary tumor-bearing animals that prevented cytostasis by reactive spleen cells.

INTRODUCTION

Infection with MTV is now thought to be a universal property of both inbred (10, 37, 41) and feral mice (35). MTV infection occurs either by genetic transmission or by ingestion of milk at birth and results in a high incidence of mammary tumors in many strains of mice. Since MTV is present very early in the life of the host, it is logical to expect that such animals may be tolerant to the virus and therefore unable to respond immunologically to MTV. Evidence suggesting the existence of tolerance to some MTV antigens has been provided by Morton (26) and Morton et al. (27). However, at least a degree of positive immune reactivity to some MTV antigens may be inferred from a number of recent reports cited below, showing that MTV-positive mice do respond immunologically to both the intact MTV and to MTV antigens on the membranes of mammary tumor cells. Pascal et al. (32) have observed subendothelial glomerular immune complex deposits containing MTV antigens in kidneys of adult Paris RIII mice carrying mammary tumors. More direct evidence for immune response to MTV has been provided by Blair et al. (3, 4), who used microcytotoxicity tests to demonstrate cytotoxic activity against MTV-positive mammary tumor cells by spleen cells from both virus-positive mouse strains as well as strains without obvious expression of MTV. These authors further showed that immune recognition and response to MTV develop within 3 weeks after birth. Humoral response to MTV in the form of precipitating antibodies has been found in a number of mouse strains (2, 16). Inhibition of macrophage migration by murine lymphocytes stimulated by MTV antigens has also recently been observed (7, 28). Lopez and Sigel (22) and Sigel et al. (39) have reported specific recognition of MTV antigens by lymphocytes from MTV-negative BALB/c mice.

Many tumor cells are resistant to cytolysis by effector cells. Therefore, when isotope release assays are used to estimate target cell-effector cell interaction, irreversible damage to target cells may go undetected. The cytostasis assay measures viability of target cells after interaction with effector cells by the ability to synthesize DNA and can therefore detect cytotoxicity in the absence of cytolysis. In this communication, we present results obtained using a cytostasis assay to study the immune response to MTV antigens expressed on the membranes of tissue-cultured mammary tumor cells. In addition to defining some of the cellular mechanisms required for immune response to MTV, we also investigated the development of natural immunity to MTV infection. It was apparent that mammary tumors are induced and progress despite significant immunity to MTV.

MATERIALS AND METHODS

Animals. BALB/c, C3H/He, C3Hf X C57B1. NIH Swiss, and athymic nude mice were obtained from the NIH breeding colony. RIII mice were bred from stock kindly furnished by Dr. Dan Moore (New Jersey Institute for Medical Research, Camden, N. J.). The RIII and C3H/He strains are heavily infected with type B particles and express high titers of MTV antigens in milk. The other strains used in our experiments were without obvious infection with MTV. The NIH Swiss and nude mice were outbred. The foster-nursed C3H mice exhibit MTV antigens in milk samples taken late in life and only at high (>4) parity levels. Animals were
immunity to MTV

housed in individual quarters with air-handling systems designed to eliminate the danger of cross-contamination of animals within the colony. The colony was serologically tested every 2 months by Dr. John C. Parker (Microbiological Associates, Inc., Bethesda, Md.). During the experiments, the only evidence of indigenous mouse virus was a declining level of antibody to murine parvovirus in the RIII colony (<10% positive).

**Tissue Culture Cell Lines.** The following tissue culture cell lines were obtained through the kindness of Dr. Wade Parks (National Cancer Institute, Bethesda, Md.): MTV-producing line C3H MT originally isolated by Owens and Hackett (29), high-MTV-positive cell line 341 clone 101, and low-MTV-expressing line LB1E. The RIII MT tissue culture cell line was obtained from Dr. Etienne Lasfargues (New Jersey Institute for Medical Research). All cells were cultured on Dulbecco-Vought medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units of penicillin and 100 µg streptomycin per ml. All cell lines were periodically checked for Mycoplasma contamination and were consistently Mycoplasma negative.

MTV-negative cell lines BALB/3T3 and LM fibroblasts were also kindly provided by Dr. Wade Parks. Culture medium and conditions were as described above.

**Immunofluorescence Test.** The indirect immunofluorescence test using viable cells was a modification of the method described by Tevethia et al. (40). Cell monolayers were dispersed with 0.2% trypsin after being rinsed with Hanks' balanced salt solution. Tissue culture medium with serum was then added to prevent further trypsinization. The cells were centrifuged and the cell pellets were resuspended in PBS, pH 7.2, and counted; portions containing 1 to 3 x 10⁶ cells were then dispersed into small centrifuge tubes. After centrifugation at 150 x g for 10 min, the PBS was removed from the cell pellets and the cells were resuspended in 0.1 ml of rabbit anti-MTV serum diluted 1:5 in PBS before use. Details regarding the preparation and specificity of the anti-MTV serum may be found in Ref. 10. Briefly, anti-MTV serum did not bind significantly to cells expressing murine leukemia virus. Immunofluorescence staining of MTV-positive mammary tumor cells was observed after absorption in vivo in weanling C57BL mice.

**Cytostasis Assay.** The cytostasis assay used was a modification of the method described by Chia and Festenstein (5). Briefly, the assay was performed as follows: varying ratios of target and effector cells were seeded in culture tubes. The cultures were incubated at 37° in an atmosphere of 5% fetal bovine serum, 100 units of penicillin per ml, and 100 µg of streptomycin per ml. After 48 hr in vitro, the cultures were swirled to resuspend the lymphocytes. The culture fluids with the lymphocytes were decanted and discarded. The target cell cultures were then recharged with fresh medium containing 1 µCi of [³H]thymidine per ml (specific activity, 0.5 Ci/m mole). [³H]Thymidine was purchased from New England Nuclear, Boston, Mass. Incubation at 37° continued for an additional 20 hr. The cultures were then washed 3 times and the cells were lysed by the addition of 0.25 ml of distilled water. The lysate was then solubilized using NCS solubilizer (Amersham/Searle, Downers Grove, Ill.) and the content of [³H] was determined using standard liquid scintillation methods and a Beckman L230 liquid scintillation spectrometer. Results were expressed as cpm ± 1 S.D.

**Preparation of Effector Cells.** Effector cells were obtained from lymphoid organs pooled from panels of 3 to 4 donor mice. Unless otherwise specified, effector cell donors were 2 to 3 months old. Spleen and thymus cells were prepared by gentle teasing. Lymph node cells were brought into suspension by pressing pools of axillary, brachial, inguinal, and mesenteric nodes through a fine nylon mesh. Bone marrow cells were expressed from the long bones of donors under gentle pressure using a fine-gauge needle. The cells were washed twice before use. Percentage of viable cells was determined using the trypan blue exclusion method. Details of these techniques are described in Ref. 8.

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Preparation of MTV. MTV was extracted from RIII mouse milk. RIII milk (50 ml) was diluted 2-fold with 1 mM Tris-HCl (pH 7.0) containing 15 mM EDTA and 150 mM NaCl and homogenized at 4°C in a glass Dounce-type homogenizer. Diluted skimmed milk was prepared by low-speed centrifugation (10,000 × g for 10 min) and then centrifuged at 92,000 × g for 1.5 hr through a 20% sucrose gradient in a Beckman Ti-14 rotor, and the resulting pellet was resuspended in 0.05 M Tris-HCl (pH 7.0) and stored frozen at −70°C until use. The purified MTV was disrupted with Tween 80 and diethyl ether (36) prior to use in the blocking experiments.

Electron Microscopy. Estimation of the expression of type B particles in tissue-cultured cells was performed exactly as described previously (10).

Statistical Analysis. The data were analyzed statistically using Student’s t test.

RESULTS

Fluorescence Assay of the Cell Lines Used. The percentage of cells expressing MTV antigen on their membranes was determined for each of the tissue culture cell lines used in these studies. Results are provided in Table 1 and confirm the presence of high percentages of cells bearing MTV cell surface antigens in the cell lines derived from mammary adenocarcinomas. 3T3 and LM cells were found to be negative. Similar results were reported in Ref. 10.

Specificity of the Cytostasis Assay Using Spleen Cells. Preliminary experiments suggested that addition of RIII spleen cells to cultures of RIII MT cells resulted in cytostasis of target RIII MT cells.

Table 2 confirms that spleen cells from both inbred and outbred animals caused cytostasis of MTV-positive RIII MT and C3H MT cells. MTV-negative 3T3 cells were not suppressed by the same spleen cell preparations while a significant feeder effect was observed with LM cells. Spleen cells from immunodeficient athymic donors or newborn donors were unreactive to MTV-positive tumor cells.

The results presented in Table 2 also demonstrated that histocompatibility differences did not significantly influence the results, since cytostasis by allogenic spleen cells was not significantly greater than that caused by syngeneic effector cells.

Blocking of Cytostasis with MTV. Specificity of the reaction of RIII spleen cells to RIII MT Cells was also demonstrated by blocking experiments (Table 3). Tween 80-ether-disrupted MTV purified from RIII milk was added to the tissue culture medium. As can be seen, cytostasis of RIII MT cells caused by RIII spleen cells was significantly blocked when MTV antigens were present in the culture fluid. The same disrupted MTV preparation had been previously shown to induce DNA synthesis in lymphocyte cultures prepared from the spleens of adult RIII donors (7); newborn spleen cells were unreactive under identical conditions. Blocking of cytostasis was also attempted using disrupted murine leukemia virus. A slight reduction of cytostasis (10% of that observed with disrupted MTV) was observed in 1 of 3 experiments. The conclusion was drawn that the cytostasis was almost exclusively a function of immune reactivity to type B virus and that the low level of type C viral antigens (30) produced by the target cell lines in vitro did not influence the results.

Optimal Ratio of Target to Effector Cells. Experiments were performed to determine the optimal ratio of target to effector cells. Typical results are illustrated in Table 4.

Table 1
Percentage of immunofluorescence-positive cells using anti-MTV serum

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Electron microscopically virus expression</th>
<th>Mean % immunofluorescence positive</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H MT</td>
<td>+</td>
<td>44</td>
<td>56-32</td>
</tr>
<tr>
<td>RIII MT</td>
<td>+</td>
<td>25</td>
<td>39-19</td>
</tr>
<tr>
<td>341 Clone 101</td>
<td>+</td>
<td>70</td>
<td>85-68</td>
</tr>
<tr>
<td>LB1E</td>
<td>+</td>
<td>11</td>
<td>12-8</td>
</tr>
<tr>
<td>BALB/3T3</td>
<td>–</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>LM</td>
<td>–</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Table 2
Cellular specificities of cytostasis assay

<table>
<thead>
<tr>
<th>Effector cell*</th>
<th>3T3a</th>
<th>RIII MT</th>
<th>C3H MT</th>
<th>LM</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIII adult spleen</td>
<td>6,221 ± 1,410</td>
<td>53,841 ± 5,722</td>
<td>116,901 ± 15,898</td>
<td>10,157 ± 1,168</td>
</tr>
<tr>
<td>C3H adult spleen</td>
<td>7,438 ± 1,534</td>
<td>9,532 ± 1,044</td>
<td>34,495 ± 2,111</td>
<td>22,687 ± 3,210</td>
</tr>
<tr>
<td>Nu/Nu adult spleen</td>
<td>6,609 ± 1,163</td>
<td>6,831 ± 1,126</td>
<td>29,874 ± 5,502</td>
<td>19,905 ± 2,852</td>
</tr>
<tr>
<td>C3H newborn spleen</td>
<td>9,422 ± 873</td>
<td>10,171 ± 942</td>
<td>35,934 ± 4,018</td>
<td>13,732 ± 1,076</td>
</tr>
<tr>
<td>RIII newborn spleen</td>
<td>10,051 ± 307</td>
<td>48,114 ± 4,935</td>
<td>140,196 ± 20,557</td>
<td>16,684 ± 2,280</td>
</tr>
</tbody>
</table>

* Effector to target cell ratio was 100:1 in all cases.

The results presented in Table 2 also demonstrated that histocompatibility differences did not significantly influence the results, since cytostasis by allogenic spleen cells was not significantly greater than that caused by syngeneic effector cells.
Blocking of the cytostatic activity of spleen cells by addition of disrupted MTV

<table>
<thead>
<tr>
<th>Effector cells*</th>
<th>+ RIII spleen + MTV*</th>
<th>Target-effector cell ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment</td>
<td>Target cell no.</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>5 x 10⁴</td>
<td>45.80 ± 5.247</td>
</tr>
<tr>
<td></td>
<td>1 x 10⁴</td>
<td>56.078 ± 3.977</td>
</tr>
<tr>
<td>2</td>
<td>5 x 10⁴</td>
<td>54.688 ± 4.532</td>
</tr>
<tr>
<td></td>
<td>1 x 10⁴</td>
<td>39.110 ± 6.276</td>
</tr>
</tbody>
</table>

* [³H]Thymidine uptake by RIII MT cells after incubation with 1 x 10⁶ RIII spleen cells. Culture and assay conditions as described in “Materials and Methods.”

Table 4

<table>
<thead>
<tr>
<th>Target cell no.</th>
<th>% suppression*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10⁴</td>
<td>77.8</td>
</tr>
<tr>
<td>5 × 10⁴</td>
<td>86.7</td>
</tr>
<tr>
<td>1 × 10⁴</td>
<td>89.5</td>
</tr>
<tr>
<td>5 × 10⁴</td>
<td>76.3</td>
</tr>
<tr>
<td>1 × 10⁴</td>
<td>55.1</td>
</tr>
</tbody>
</table>

* Percentage of suppression = 100 – cpm target and effector cells / cpm target cell

Table 5

<table>
<thead>
<tr>
<th>Target cell no.</th>
<th>None</th>
<th>+ CH3 (low)</th>
<th>+ CH3 (high)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10⁴</td>
<td>63.226 ± 1.103</td>
<td>12.077 ± 1.004</td>
<td>27.516 ± 1.904</td>
</tr>
<tr>
<td>5 × 10⁴</td>
<td>26.731 ± 2.312</td>
<td>9.117 ± 846</td>
<td>22.140 ± 1.369</td>
</tr>
<tr>
<td>1 × 10⁴</td>
<td>17.560 ± 1.467</td>
<td>6.174 ± 515</td>
<td>10.334 ± 916</td>
</tr>
</tbody>
</table>

* [³H]Thymidine uptake by CH3 MT cells after incubation with 1 × 10⁶ C3H spleen cells. Culture and assay conditions as described in “Materials and Methods.”

Spleen cells (1 × 10⁴) from adult RIII donors were incubated with varying numbers of RIII MT Cells. As can be seen, when the number of spleen to target cells was 10:1 or more the uptake of DNA precursor by RIII MT cells was significantly reduced. Since optimal results were observed with a ratio of 100 effector cells to 1 target cell, this ratio was used in most of the later experiments.

Effectiveness of Spleen Cells from High- and Low-MTV-expressing Donors. Spleen cells from C3H donors expressing either high or low levels of MTV [measured in milk samples taken at the 1st 3 parturitions using a hemagglutination inhibition assay (10)] were also tested in the cytostasis assay. The results are illustrated in Table 5. Both MTV-negative and -positive spleen cells were reactive to the C3H MT target cells. However, as can be seen from the table, spleen cells from donors expressing low levels of MTV were significantly more cytostatic to C3H MT cells than splenocytes from animals highly positive for MTV.

Cytostasis in High- and Low-MTV-expressing Target Cells. The question was asked whether spleen cells from an individual source would be more reactive to cells producing high levels of MTV in vitro than to cells from clones derived from the same parent line but expressing only very low levels of MTV. Results obtained using 341 clone 101 and LB1E cells are provided in Table 6. Spleen cells from normal RIII donors were significantly more cytostatic to the high-MTV-producing 341 clone 101 cells than to LB1E cells.

Ability of Other Lymphoid Cell Types to Function in the Cytostasis Assay. Other kinds of effector lymphoid cells were tested in the cytostasis assay using C3H MT cells. It was observed that lymph node cells were approximately as active as spleen cells (Table 7). PEC were the most cytostatic population tested. Only minimal cytostasis occurred when thymocytes or bone marrow cells were assayed.

Role of Adherent Cells in Cytostasis. Adherent cells were purified as described in “Materials and Methods.” C3H MT cells were then added to the adherent cells. Virtually all of the cytostatic activity of spleen cells was retained by the

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nonadherent population of the spleen. The reverse was true when PEC were fractionated by adherence, i.e., only the adherent PEC were cytostatic toward C3H MT cells.

Adherent cells have been shown to have a regulatory role in the spleen (6, 31), i.e., removal of adherent cells enhanced the splenic response to mitogens. To test further whether splenic adherent cells may influence the response of nonadherent RIII spleen cells to RIII MT cells, the following experiments were performed. Adherent cells were prepared by seeding culture tubes with 1, 2, and 4 times the normal number of spleen cells. Fourteen hr later, the nonadherent cells were removed and the nonadherent cells from 1 x 10^6 spleen cells were added along with 1 x 10^6 RIII MT cells. This procedure increases the ratio of adherent to nonadherent cells. Increasing the numbers of splenic adherent cells only slightly reduced the suppressive effects of RIII spleen cells upon DNA synthesis by RIII MT cells in vitro.

The conclusion was drawn that splenic adherent cells do not regulate cytostatic activity of nonadherent spleen cells.

**Effect of T-lymphocyte Depletion.** RIII spleen cells were depleted of θ-bearing lymphocytes by treatment with anti-θ serum (Table 8). It was found that depletion of T-lymphocytes did not change the reactivity of RIII spleen cells to cultured RIII MT cells. These results indicate that the effector cell is likely to be the Ig-bearing B-lymphocyte or possibly the null cell. Experiments designed to answer this question are in progress.

**Blocking Factors.** Factors present in the serum of tumor-bearing animals can block the interaction between lymphoid cells and target tumor cells. Serum from tumor-bearing C3H mice was compared with serum from normal C3H mice of equivalent age in the cytostasis assay. Table 9 demonstrates that addition of serum from tumor-bearing C3H mice at low percentages (<2%) suppressed cytostasis of C3H MT cells by C3H spleen cells. Serum from normal syngeneic donors of approximately the same age was not cytostatic when added to the culture medium at comparable levels. Whether the factors responsible for blocking are antibodies or complexes of MTV antigen with antibody remains to be determined.

## DISCUSSION

A cytostasis assay for immunity to MTV antigens on the membranes of mammary tumor cells has been used to demonstrate immunological reactivity of normal mice to MTV. The results confirm immune sensitivity to MTV antigens in all but neonatal mice or T-lymphocyte-deficient nude mice. While all strains of mice exhibited some type of immune recognition of MTV-positive target cells, spleen cells from high-MTV-expressing animals were significantly less cytostatic than similar cells from low expressor donors. Specificity was demonstrated by blocking cytostasis by soluble MTV antigens and by the fact that MTV-negative target cells were unaffected by the presence of MTV immune effector cells. In addition, effector cells were more cytostatic to cultured cells producing high levels of MTV than to cells expressing only minimal amounts of virus.

Depletion of T-lymphocytes did not affect cytostasis of MTV-positive cells by spleen cells. On the other hand, athymic nude mice were unresponsive to MTV-positive cells. These data suggest, therefore, that initiation of immunity to MTV cell surface antigens probably requires cooper-
ation with T-lymphocytes. However, the final effector cell that is cytotoxic to MTV-positive target cells appears to be a non-T-lymphocyte, since treatment with anti-θ serum did not reduce cytostatic activity. Similar conclusions have been drawn by Kearney et al. (18), who found that treatment with anti-θ serum interfered with the early phase of cellular immunity to tumors. The late phase was unaffected by lysis of T-lymphocytes by anti-θ serum, while removal of B-lymphocytes resulted in abrogation of immunity in both early and late phases.

An additional factor influencing the results may be the presence of immunoreactive null cells in the effector cell preparations. Kiessling et al. (19) have shown that “natural” cytotoxic lymphocytes exist in some mouse strains that have the capacity to lyse tumor cells. The effector cell in this system was neither θ positive nor Ig bearing (20), i.e., a null lymphocyte. Null cells may interact with MTV-positive cells and thereby cause at least part of the observed cytostasis. Natural cytotoxicity toward syngeneic and allogeneic tumors associated with type C viruses has been recently reported by Herberman et al. (14). This naturally occurring effector cell appears also to be a null lymphocyte (13).

Histoincompatibility did not influence cytostasis for the following reasons: (a) when target and effector cells were syngeneic, cytostasis was about equal to that found in allogeneic systems. (b) the response by effector cells occurred within 48 hr. Sensitization to alloantigens occurs in vitro but requires longer for expression (12).

PEC were found to be highly active effector cells in the cytostasis assay. It seems likely that the reaction of PEC was fundamentally different from that of spleen or lymph node cells. PEC induced by injection of irritants, such as adjuvant or proteose peptone, have been shown to be hyperreactive to target antigens (11) or cells (15, 17) but in a nonspecific fashion. Mackaness (24) has shown that stimulated macrophages are hyperreactive to Listeria monocytogenes when produced in this manner and can nonspecifically protect against infection with this organism. Similar protection against tumor cell challenge results from stimulation of the reticuloendothelial system by Bacillus Calmette-Guérin (43).

Nonadherent PEC were inactive in the cytostasis assay despite the presence of T- and B-lymphocytes. These data imply that fundamental differences may exist between lymphoid cells from the peritoneum and spleen, at least in terms of functional capacities. Cytotoxicity found exclusively in the adherent cells has been reported by Meltzer et al. (25), who studied peritoneal cells from mice infected with Mycobacterium bovis. Krahnenbuhl and Remington (21) observed antitumor cell activity in adherent peritoneal macrophages while peritoneal lymphocytes were inactive. Anti-tumor activity by peritoneal lymphocytes has been reported by Berke et al. (1) after immunization with allogeneic EL4 tumor cells.

The role of adherent cells in the regulation of the splenic cytostatic activity was also studied. Unlike mitogen responsive cells (6), spleen cell cytostasis was unchanged by addition of increased numbers of adherent cells to the reaction mixture.

Progressively growing MTV-positive tumors appear to elicit formation of blocking factors. When serum from tumor-bearing donors was added to the culture medium, a significant part of spleen cell-induced cytostasis was eliminated. Whether the blocking was due to antibody coating of the target tumor cells or to the presence of antigen-antibody complexes is not apparent from the data. Our observations are consistent with those of Pollack et al. (33), who found that serum-mediated cytotoxicity could only be produced by sera from donors whose MTV-induced adenocarcinomas had been excised. Serum from murine sarcoma virus-tumor-bearing mice has been shown to inhibit cytostasis of lymphoma by spleen and lymph node cells (38).

REFERENCES


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