Subset of Spleen Lymphocytes from BALB/cCrgl Mice Stimulated by Mouse Mammary Tumor Virus

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ABSTRACT

Lymphocytes from BALB/cCrgl mice react to mouse mammary tumor virus-associated antigen(s) when tested in in vitro blastogenic transformation assays. These mice have a low incidence (<1%) of spontaneous mammary tumors and are free from complete mammary tumor virions. We have studied the nature of the lymphoid cells mediating the lymphocyte transformation reaction to purified mammary tumor virus. With the use of nylon wool columns, the responder cells were found to belong to the nylon-adherent population. The T-lymphocytes were not stimulated by mammary tumor virus even in the presence of added macrophages. These results were confirmed with treatments of spleen cells with either anti-surface immunoglobulin and complement or anti-Thy 1 antigen and complement. Thus, B-cells seem to be the lymphoid population responsive to mammary tumor virus-associated antigen(s) in the spleen of BALB/cCrgl. The cause of this reactivity may be a result of any of the following: (a) horizontal transmission; (b) activation of spleen cells by viral cell contaminants in mammary tumor virus preparations; (c) a nonspecific mitogen reaction exerted by the virus in the system; or (d) sensitization to mammary tumor virus-associated antigen(s) due to the expression of an endogenous virus. We present here data arguing against the first three possibilities. In recent work, we found evidence supporting the expression of mammary tumor virus-related antigen(s) on lymphoid cell surfaces of BALB/cCrgl. From these studies, we propose that the responses seen in our in vitro assays may represent a sensitization event resulting from exposure to an endogenous mammary tumor virus gene product.

INTRODUCTION

The role of either cell-mediated or humoral immunity in mammary tumorigenesis has never been well defined, in part due to the multifactorial etiology of breast cancer. Blair et al. (2) observed that BALB/c adult females free of detectable MMTV³ particles possessed spleen cells reactive against MMTV-induced mammary tumor cells. Gillette and Lowery (9), using a cytostasis assay, investigated natural reactivity to MMTV antigen(s). They found that spleen cells from adults of BALB/c, C3H/He, and C3Hf x C56BL were cytostatic to a variety of MMTV-positive mammary tumor cell lines. In addition, the degree of reactivity of spleen cells from low MMTV expressor was higher than that of the spleen cells of syngeneic high MMTV expressor.

In our laboratories, we have found that early in life, BALB/c mice respond to MMTV-associated antigen(s) in lymphocyte transformation assays (18, 27). Animals bearing large MMTV-negative chemically induced mammary tumors (D1-DMBA-3) have depressed reactivities to mitogens but behave in a similar manner to that of normal BALB/c in respect to their blastogenic responses to MMTV-associated antigen(s). The specificity of the reaction was ascertained by several parameters (21). Thus, preincubation of MMTV with anti-MMTV serum inhibited the blastogenic reaction. Antibody against Rauscher leukemia virus had no inhibitory effect and did not induce positive reactions by itself or in the form of antigen-antibody complex with the homologous Rauscher leukemia virus.

In the present investigation, using separated splenic lymphocyte populations, we report that B-lymphocytes are the cells responding to the viral-associated antigen(s) in lymphocyte transformation assays in the low MMTV expressor mouse strain BALB/cCrgl.

MATERIALS AND METHODS

Animals and Tumors. Fourteen- to 18-week-old BALB/c virgin mice bred in our laboratories by sister-brother matings were used throughout the studies. The colony is housed by itself, in quarters physically separated from other animals. Their incidence of spontaneous mammary tumors is less than 1% during their first year of life.

The syngeneic transplantable mammary tumor designated as D1-DMBA-3 was kindly provided by Dr. P. Blair (University of California) and was maintained in our laboratories by s.c. injections of minced tumor preparations. The tumor originated from a hormonally stimulated preneoplastic nodule transplanted to a mouse treated with 7,12-dimethylbenzanthracene (23) and has been found consistently to be devoid of complete MMTV particles by means of electron microscopy, immunofluorescence, and radioimmunoassays.⁴

Virus. MMTV purified from RII milk by a combination of rate zonal and isopyknic centrifugation was kindly provided by the Virus Cancer Program from stocks supplied by the Meloy Laboratories, Inc., Springfield, Va. MMTV of RII origin obtained from virus-infected culture fluids of Crandall feline kidney cells was generously donated by Dr. Jeffrey Schlam, National Cancer Institute, NIH. The virions were purified as described by Teramoto et al. (29).

Preparation of Spleen Cells. Spleens were removed asep-
Pretically from normal and tumor-bearing mice. The cells were passed through a stainless steel mesh with siliconized rubber stops and washed through with 10 ml of HBSS. To obtain single cells, clumps were removed by filtering through loosely packed nylon fibers. The resulting suspension was centrifuged, and the cell pellet was subjected to a hypotonic shock for elimination of contaminating RBC. A second passage through a nylon sieve removed the remaining clumps. The cells were washed in HBSS resuspended in Roswell Park Memorial Institute Tissue Culture Medium 1640 with 40% fetal bovine serum and incubated for 1 hr at room temperature on glass surfaces to remove adherent cells. A count was performed to determine the number of lymphocytes after washing the cells in HBSS. With this procedure, the final cell population contained mainly T (44% 1-positive)- and B (45% Slg-positive)-lymphocytes. Macrophages were reduced to less than 2% as indicated by a lipase staining (16, 25) and ingestion of carboxyl iron. No remaining granulocytes could be detected with Wright-Giemsa staining.

Preparation of Peritoneal Exudate Macrophage Populations. PEC were induced in normal mice by i.p. injection of 2 ml of 1% oyster glycogen, 24 hr prior to harvest as described previously (18). Under these conditions, 90% of the phagocytic cells were found to be macrophages by morphology and lipase staining.

Nylon Wool Column Purification. The method described by Julius et al. (14) was used with the modifications described by Epstein et al. (8). The resulting nylon-nonadherent and nylon-adherent populations were characterized respectively as T- and B-lymphocytes by means of cell surface markers, i.e., presence of Thy 1 antigen or Slg and by functional tests.

Detection of Surface Immunoglobulin and Thy 1 Antigen-bearing Cells. FITC-conjugated rabbit anti-mouse IgG F(ab')2 fragments (Cappel Laboratories, Cochranville, Pa.) were used in the direct immunofluorescence assay to detect surface immunoglobulins. The procedure used was that described by Rabellino et al. (26). An indirect immunofluorescence technique using rabbit anti-Thy 1 serum (Cedar Lane Laboratories, Hicksville, N. Y.), and FITC goat anti-rabbit IgG F(ab')2 fragments was used to detect Thy 1-positive cells. Details of these procedures have been published previously (4, 8).

Lymphocyte Transformation Assay. The various lymphocyte fractions were centrifuged and adjusted to a concentration of 1 x 10⁶ cells/ml in the growth medium described by Click et al. (5). Functional tests were performed using PHA (Wellcome Research Laboratories, Beckenham, Kent, England) and Con A (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) as T-cell mitogens and LPS (Difco Laboratories, Detroit, Mich.) as B-cell mitogen. MMTV and mitogen dilutions were added in 25-μl volumes at the concentrations shown in the charts and tables. Cultures were maintained for 3 days in microculture plates (Linbro; Flow Laboratories, Inc., Rockville, Md.) with 1 x 10⁶ cells/well and were given an 18-hr pulse of tritiated thymidine (0.5 μCi/well; Amersham/Searle Corp., Arlington Heights, Ill.). The cells were collected on glass fiber filter paper with distilled water in a SKATRON multiple automated sample harvester. The filters were counted for radioactivity as described previously (22), and results were expressed as cpm.

Treatment of Spleen Cells with Anti-Thy 1 or Anti-mouse Slg and Complement. Rabbit anti-Thy 1 antibody was purchased from Accurate Chemicals (Hicksville, N. Y.). Goat anti-Slg was kindly provided by the Virus Cancer Program from stocks supplied by the Frederick Cancer Research Center. The spleen cells prepared as described above were incubated for 1 hr at 4° with a final dilution of 1:10 of anti-Slg and 1:20 of anti-Thy 1. The cells were washed twice in Roswell Park Memorial Institute Tissue Culture Medium 1640, and 1:10 final dilutions of Low-tox guinea pig complement (Cedar Lane Laboratories) were added. The mixtures were incubated at 37° for 1 hr. Several controls were included in all experiments, i.e., cultures with complement alone and anti-Slg or anti-Thy 1 in the absence of complement. None of the control treatments were toxic to the lymphocytes. Significant depletion of B-lymphocytes (down to 7% Slg positive) was obtained after treating with anti-Slg and complement, while there was an enrichment of these cells (up to 74% Slg positive) after anti-Thy 1 and complement treatment. The cells subjected to the various treatments were washed extensively and resuspended to the cell concentrations appropriate for the blastogenic assays.

RESULTS

Stimulation of spleen cells from BALB/cCr1g1 mice can be observed after incubation with various concentrations of MMTV purified from RIIL milk. Since these preparations might contain small amounts of milk antigens that could be contributing to the observed response, experiments were performed using MMTV grown in infected Crandall feline kidney cells as antigenic source. Chart 1 shows that similar responses can be obtained in the blastogenesis assay when one stimulates the lymphocytes with either MMTV from milk sources or MMTV derived from the supernatants of cat kidney cells. Culture fluids of noninoculated Crandall feline cells failed to elicit any enhanced incorporation of [³H]thymidine at any concentration, indicating that the responsiveness of the spleen lymphocytes of the BALB/cCr1g1 mice is not due to a nonspecific mitogenic
A study was undertaken to determine the responder cells involved in the blastogenic reaction to MMTV. In order to ascertain the efficacy of the nylon wool separation technique, cell surface marker studies were performed in the cell preparations. Table 1 shows the percentages of Sig-positive and Thy 1-positive cells. It can be seen that Sig-positive cells are found mainly in the nylon-adherent populations in both normal and tumor-bearing mice while the nylon-nonadherent lymphocytes express Thy 1 antigen. Functional tests (Table 2) using B- and T-cell mitogens in the lymphocyte transformation assay revealed that responses to the T-cell mitogens PHA and Con A are mainly located in the nylon-nonadherent fractions while responses to the B-cell mitogen LPS is confined to the nylon-adherent populations. Unseparated spleen cells from normal and D1-DMBA-3 tumor-bearing BALB/cCrIgI mice had similar rates of synthesis of DNA when stimulated with purified MMTV (Chart 2). Nylon-nonadherent cells did not significantly respond to viral components. In sharp contrast, nylon-adherent spleen cells were capable of being stimulated by MMTV antigen(s) in the lymphocyte transformation assay.

It could be argued that the lack of responses of the T-lymphocytes to viral antigens may be due to the absence of the small percentages of macrophages (<1%) that can be detected by lipase staining in our nylon-adherent population. These cells might be necessary for eliciting positive blastogenic responses in the T-cell population (12). Thus, experiments were set up running parallel cultures of various lymphocyte subpopulations supplemented with macrophage-rich PEC. In Chart 3, it can be seen that addition of $1 \times 10^5$ PEC to the nylon column-separated populations exerted no significant enhancement on the responses of either B- or T-cells to MMTV-associated antigen(s), although there was an increase in the cpm incorporated by the unstimulated cultures when PEC were

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**Table 1**  
Cell surface markers on nylon wool-separated spleen cells from normal and D1-DMBA-3 tumor-bearing BALB/cCrIgI mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Cell preparation</th>
<th>Thy 1</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal BALB/cCrIgI</td>
<td>Unseparated</td>
<td>44 ± 6.8(^a)</td>
<td>42 ± 7</td>
</tr>
<tr>
<td></td>
<td>Nylon nonadherent</td>
<td>97 ± 3</td>
<td>4 ± 3</td>
</tr>
<tr>
<td></td>
<td>Nylon adherent</td>
<td>2 ± 2</td>
<td>89 ± 4</td>
</tr>
<tr>
<td>D1-DMBA-3 tumor bearers(^b)</td>
<td>Unseparated</td>
<td>45 ± 5</td>
<td>48 ± 2</td>
</tr>
<tr>
<td></td>
<td>Nylon nonadherent</td>
<td>90 ± 2</td>
<td>3 ± 2</td>
</tr>
<tr>
<td></td>
<td>Nylon adherent</td>
<td>1</td>
<td>91 ± 5</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± S.D.  
\(^b\) Animals tested when tumors were small (1 to 2 g).

**Table 2**  
Functional tests in nylon wool-separated cells using the lymphocyte transformation assay

<table>
<thead>
<tr>
<th>Mice</th>
<th>Cell preparation</th>
<th>Nonstimulated cultures</th>
<th>PHA (1:800)</th>
<th>Con A (1 (\mu)g)</th>
<th>LPS (25 (\mu)g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal BALB/cCrIgI</td>
<td>Unseparated</td>
<td>829 ± 198(^a)</td>
<td>25,875 ± 1,983</td>
<td>49,641 ± 2,723</td>
<td>8,971 ± 8897</td>
</tr>
<tr>
<td></td>
<td>Nylon nonadherent</td>
<td>- 627 ± 109</td>
<td>36,436 ± 4,432</td>
<td>68,730 ± 3,941</td>
<td>898 ± 91</td>
</tr>
<tr>
<td></td>
<td>Nylon adherent</td>
<td>989 ± 187</td>
<td>3,143 ± 297</td>
<td>5,995 ± 1,348</td>
<td>9,873 ± 1,093</td>
</tr>
<tr>
<td>D1-DMBA-3 tumor-bearing BALB/cCrIgI</td>
<td>Unseparated</td>
<td>1,193 ± 283</td>
<td>29,413 ± 2,144</td>
<td>58,499 ± 4,712</td>
<td>9,491 ± 1,231</td>
</tr>
<tr>
<td></td>
<td>Nylon nonadherent</td>
<td>761 ± 138</td>
<td>43,747 ± 5,001</td>
<td>74,343 ± 6,139</td>
<td>699 ± 60</td>
</tr>
<tr>
<td></td>
<td>Nylon adherent</td>
<td>1,214 ± 823</td>
<td>3,146 ± 298</td>
<td>6,916 ± 579</td>
<td>11,532 ± 1,343</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± S.D.  
\(^b\) Animals tested when tumors were small (1 to 2 g).
added. Similar experiments not presented in this chart were performed using varying ratios of PEC to lymphocytes with the same results. In addition, no responses were detected in control cultures containing MMTV and macrophage populations without addition of lymphocytes.

The results obtained with the nylon wool-separated spleen cells were reconfirmed in depletion studies. Treatment of unseparated splenocytes with anti-Slg and low-tox guinea pig complement (Chart 4) resulted in decreased responses to LPS, a B-cell mitogen, and augmented responses to Con A, a T-cell mitogen, indicating enrichment of T-lymphocytes. Conversely, treatment with anti-Thy 1 and complement resulted in diminished response to Con A and heightened response to LPS, evidence of an enrichment of B-lymphocytes. Treatments with anti-Thy 1.2 and complement resulted in higher levels of responses to MMTV antigen(s) in the lymphocyte transformation assays. In contrast, there was a dramatic decrease in the MMTV-induced blastogenic reaction upon treatment of lymphocytes with anti-Slg and complement. These results provide further indication that the reactive cells involved in the in vitro responsiveness to MMTV belong to the B-lymphocytes.

**DISCUSSION**

Previous studies in our laboratories have disclosed that BALB/cCrgl mice respond to MMTV-associated antigen(s) in 2 parameters of cell-mediated immunity: blastogenic transformation and migration inhibition (18, 21). In contrast with the results obtained when tumor antigen(s) are used as antigenic stimulus (19, 22), the responsiveness to MMTV-associated antigen(s) is not affected by the presence of large tumors. Thus, lymphocytes from BALB/cCrgl mice implanted with D1-DMBA-3 tumors gave similar responses in the lymphocyte transformation assay as those from normal BALB/cCrgl mice. In the present report, we have analyzed the spleen lymphocytes reactive in blastogenic transformation assays to purified MMTV. Nylon wool fractionation was used to determine which cells are involved in this reaction. This procedure has been used for several years in our laboratories, and in our hands it yields highly enriched T- and B-cell populations (4, 7, 20).

Using these nylon-separated populations, we now report that B-lymphocytes are the cells responding in lymphocyte transformation assay to MMTV antigen(s). No responses were detected in the nylon-nonadherent lymphocyte populations. The validity of these results were further ascertained by means of depletion studies using anti-Slg and anti-Thy 1 sera and complement. In addition, we have observed that these B-lymphocytes when stimulated in vitro with MMTV will differentiate into blast and plasma cells as detected in morphological studies using Wright-Giemsa stains.6

Lane et al. (15) analyzed the effector cells responsible for cytotoxicity against MMTV-expressing mammary tumor cells. They concluded that both T-cell-mediated and non-T-cell-mediated killing of MMTV-induced target cells can be detected in BALB/cfC3H (BALB/c mice foster nursed on C3H mice), but the supposedly MMTV-free BALB/c mice show a cytotoxic activity that does not involve T-lymphocytes. It should be pointed out that the effector cells of the cytotoxic reactions are not necessarily the same as those involved in the lymphocyte transformation assay.

Gillette and Lowery (9), with a cytostasis assay, tested lymphocytes from several strains of mice using MMTV-expressing mammary tumor cells as targets. In these experiments, they found considerable reactivity in spleen cell preparations from low MMTV expressor strains against the MMTV-positive cells. These investigations also observed that the degree of reactivity to the target cells was not altered upon depletion of splenic T-lymphocytes by use of anti-Thy 1 serum plus complement. Thus, they concluded that the effector cells in their assays were not Thy 1-positive.

Stutman (28) measured the ability of lymphocytes from MMTV-positive C3H mice and the MMTV-negative C3Hf subline to destroy radioactively labeled mammary tumor target cells. He found that both kinds of mice exhibited primary cytotoxic responses mediated by T-lymphocytes. Moreover, the magnitude of the response in the MMTV-positive mice was lower than in the MMTV-negative strain. Upon immunization with formalinized MMTV, a moderate in vivo preventive effect was observed. In addition, a T-lymphocyte mitogenic response was observed to antigens solubilized from a DBA/2 mammary tumor and from a DBA/2 ML antigen-positive leukemia tumor line. In these studies, it appears that the response was mainly directed to cross-reacting antigens and most probably to the mammary leukemia antigen.

In our studies, we have demonstrated that BALB/cCrgl mice, a low MMTV expressor strain, possess lymphocytes capable of responding to purified MMTV preparations. There are several possible explanations for these results. (a) The animals have been infected with exogenous MMTV and have become sensitized to the viral antigens. (b) The stimulation is due to host cell contaminants of the purified viral preparations. (c) The purified MMTV is acting as a nonspecific mitogen. (d) There is an expression of an endogenous viral antigen(s) in BALB/cCrgl mice that is able to act as an immunogen to the host.

Concerning the first hypothesis, it is appropriate to reemphasize here that our colony of BALB/cCrgl mice has always been kept in an area physically separated from any other...
animals and has been handled by personnel who do not handle any other animals in our institution. In addition, the incidence of spontaneous mammary tumors in our BALB/c colony has never exceeded 1% in the first year of life. These conditions make it unlikely that horizontal transmission could have occurred, which could be the cause of MMTV recognition in in vitro assays.

The possibility that host cell contaminants in purified MMTV preparations could be stimulating the spleen cells from BALB/cCrgl mice has been addressed in the studies using MMTV from milk sources in comparison with MMTV from the same strain passaged in vitro in the Crandall feline kidney cells. Similar reactions were obtained when the spleen cells were stimulated with RIII virus purified from milk as when the RIII MMTV from the heterologous source was used.

MMTV could be causing a nonspecific polyclonal response of the B-lymphocytes of BALB/cCrgl. Induction of DNA synthesis in these lymphoid cells might be the result of MMTV acting as a nonspecific B-cell mitogen. In other studies in our laboratories, we have found indirect evidence that argues against such a possibility. Using another BALB/c mouse colony (BALB/cfC3H), derived from BALB/cCrgl animals after foster nursing at birth on MMTV-positive C3H mice, no significant responsiveness to MMTV antigens could be detected in their spleen cells, while the same preparations can be stimulated by the B-cell mitogen, LPS (17). If MMTV is acting as a nonspecific mitogen in BALB/cCrgl mice, similar reactivities should have been observed in the 2 syngeneic colonies. In addition, we have reported previously that, in animals bearing large D1-DMBA-3 mammary tumors (5 to 10 g of weight), the responses to B- and T-cell mitogen are greatly depressed (22, 27). However, in recent kinetic studies, we have found that the stimulation caused by MMTV antigen(s) remains stable in BALB/cCrgl mice even in the presence of a heavy tumor burden (17). Thus, the stimulation caused by MMTV in this mouse colony appears to be quantitatively and qualitatively different from that obtained with other mitogens such as PHA, Con A, and LPS.

In a recent report (4), we have presented evidence that there is an expression of MMTV antigen(s) in the spleen cells of BALB/cCrgl mice. Daams et al. (6), using unfixed spleen cells reacted with heterologous polyvalent anti-MMTV sera, observed a membrane immunofluorescence reaction. These initial studies only noted such reactions in high-mammary tumor incidence strains of mice (C3H, GR, and RIII) and not in a small number of low-incidence strains, perhaps due to the limited potency of the antisera. After this initial work, a number of investigators provided evidence that blood elements propagate MMTV (24). For example, lymphoid cells carrying MMTV antigen(s) include spleen cells (11, 13) and lymphocytes (3). Gillette et al. (10) conducted several experiments using indirect immunofluorescence assay and 51Cr release cytotoxicity assay to detect MMTV antigen(s) on lymphoid cells. They observed that both high- and low-mammary tumor incidence strains expressed MMTV-related cell surface antigen(s) on spleen lymphocytes. In our studies using BALB/cCrgl mice, it was found that MMTV is detectable by immunofluorescence in 24 to 34% of the spleen lymphocyte population (4). A number of controls were included in the study. FITC conjugate without any rabbit serum gave no fluorescence, and substitution of rabbit anti-MMTV serum with normal rabbit serum as a control gave minimal background fluorescence. Blocking studies with purified MMTV from milk or feline culture origin further demonstrated the specificity of the reaction. In addition, we ruled out the possibility that the immunofluorescence detected was due to binding via Fc receptors to spleen lymphocytes with the use of Fab'2 fragments of the rabbit anti-MMTV immunoglobulin and Fab'2 fragments of the fluorescein-conjugated goat anti-rabbit serum.

Taking into consideration all the facts available, it would appear that the responsiveness to MMTV observed in the spleen lymphocytes of BALB/cCrgl mice may be due to reactivity with the product resulting from expression of an endogenous MMTV viral gene(s). A similar hypothesis has been proposed by Bentvelzen (1) as a sequential activation of MMTV genes. The product(s) of such an endogenous virus could be responsible for the expression of MMTV-associated antigen(s) in the lymphoid cells of BALB/cCrgl mice and also for a putative sensitization which could account for the in vitro reactions detected.

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