In Vitro Immune Responses to Viral and Tumor Antigens in Murine Breast Cancer

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Summary

Inhibition of migration of peritoneal exudate cells proved to be a useful measurement of cell-mediated immunity which correlated in several respects with blastogenic transformation reactions.

Lectins (phytohemagglutinin and concanavalin A) inhibited the migration of peritoneal exudate cells from normal and tumor-bearing mice, whereas tumor antigen caused inhibition of migration of cells from tumor-bearing animals only. The disparity in immunogenic capacity previously observed with lymphocyte transformation studies was also manifested in migration inhibition, i.e., D1-DMBA-3 tumor being immunogenic and D1-DMBA-2 being nonimmunogenic.

Using the migration inhibition and blastogenic transformation reactions, responses were obtained to mammary tumor virus (MTV) antigen(s) in cells from BALB/cCrGl mice, which are free of MTV. In contrast, cells from MTV-positive BALB/cfC3H mice failed to respond to this antigen(s) in both reactions, suggesting a form of tolerance. However, the reactions became positive after implantation with MTV-containing spontaneous mammary tumors. Two possible explanations of the origin of reactive lymphocytes, horizontal transmission, or activation of a gene coding for an MTV antigen(s), are discussed.

Introduction

Previous studies in our laboratories have disclosed that following transplantation of syngeneic MTV-negative chemically induced D1-DMBA-3 immunogenic tumor into MTV-negative BALB/cCrGl mice their splenic lymphocytes become responsive to tumor-specific antigens (10). When BALB/c mice are implanted with a nonimmunogenic chemically induced tumor of the same series, D1-DMBA-2, their lymphocytes fail to respond to cells of this tumor or tumor extracts as measured by increased uptake of \( ^3H \) thymidine. With an increase in tumor size there is a decrease in lymphocyte response to extracts of the tumor antigen in the immunogenic tumor series and also to the mitogen, PHA, in both the immunogenic and nonimmunogenic series. This anergy could be reversed by tumor excision that can be explained in terms of relief of tumor (antigenic) burden. However, sham surgery, where the skin is cut but the tumor mass not disturbed, also restores lymphocyte responses, suggesting that nonimmunological factors might be at play (10). Lymphocytes of mice that remain tumor free after surgery retain activity to tumor antigen, whereas lymphocytes of mice in which tumors recur lose this reactivity. Thus there is a direct correlation between the immunological status as determined by the lymphocyte transformation assay and the postsurgical fate of the animal (no tumor, minimal tumor, or relapse with large or metastatic tumors).

The studies reported thus far dealt mainly with the BALB/cCrGl colony and MTV-negative chemically induced mouse mammary tumors. We now report on further findings with this system and, in addition, on studies performed on BALB/c mice of another subline to which MTV was introduced initially by foster-nursing on C3H mice at the Cancer Research Laboratories of the University of California. These mice, BALB/cfC3H, were used for transplantation with MTV-negative D1-DMBA-3 and D1-DMBA-2 tumors and MTV-positive spontaneous syngeneic tumors.

Lymphocyte Responses of BALB/c Mice to Spontaneous Mammary Tumors

Tests were performed using splenic lymphocytes of BALB/cCrGl and BALB/cfC3H mice. To ascertain whether there are antigens shared by these spontaneous tumors (presumably induced by MTV) and the MTV-negative D1-DMBA-3, both types of tumors were tested. In 3 experiments we obtained the following results (Chart 1): BALB/cfC3H mice bearing small (2-g) spontaneous mammary tumors responded to the nonspecific mitogen PHA and to crude homogenates of the autologous tumor, but not to the MTV-negative D1-DMBA-3 tumor extract; BALB/cCrGl mice bearing D1-DMBA-3 tumor reacted only to their tumor, but they failed to respond to the spontaneous tumor.

Some provisional conclusions are justified: (a) mice with spontaneous mammary tumor respond to the autologous antigen; (b) this tumor antigen does not cross-react with the antigens of the chemically induced tumors; (c) the results substantiate previous findings that the BALB/cCrGl and their tumor, D1-DMBA-3, are free of MTV at least in detectable levels.

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2 Presenter.

The abbreviations used are: MTV, mammary tumor virus; DMBA, 7,12-dimethylbenzanthracene; PHA, phytohemagglutinin; PEC, peritoneal exudate cells.
Migration Inhibition Studies of Peritoneal Exudate Cells Exposed to Mitogens and Tumor Antigens

In order to determine whether our results could be substantiated by another parameter of cell-mediated immunity, migration inhibition studies were undertaken. PEC from various groups of animals were exposed to mitogens and tumor antigens and their areas of migration were measured planimetrically. The procedures used were modifications of those described by Pomales-Lebron and Ortiz (12). The results of a series of experiments are summarized in Table 1. Only migration inhibitions of 30% or greater were considered significant. In all experiments there was a more extensive migration of cells from tumor-bearing mice compared to normal controls. Mitogens caused a significant inhibition of cells from normal mice and mice with small- or medium-sized tumors. There was no difference in the degree of inhibition when the PEC came from BALB/cCrgl or BALB/cfC3H mice. In a limited number of experiments there appeared to be a significant decrease of migration inhibition in cells from mice bearing large tumors. These results are analogous to those obtained in the blastogenic reaction, but final conclusions must await the testing of large numbers of animals.

Extracts of D1-DMBA-3 caused significant inhibition of migration of cells from BALB/cCrgl and BALB/cfC3H mice bearing D1-DMBA-3 tumors but had no effect on migration of cells of normal mice. Nonimmunogenic D1-DMBA-2 tumor extracts had no effect on migration (in keeping with their nonimmunogenic status). However, here again, the number of animals is small and additional tests are required.

<table>
<thead>
<tr>
<th>Tumor status</th>
<th>Migration areas control</th>
<th>PHA (%)</th>
<th>Concanavalin A (%)</th>
<th>D1-DMBA-3 tumor antigens (%)</th>
<th>D1-DMBA-2 tumor antigens (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/cCrgl None</td>
<td>1.73</td>
<td>60 (18)</td>
<td>43 (14)</td>
<td>0 (8)</td>
<td>10 (2)</td>
</tr>
<tr>
<td>Small (0.1–2.5 g)</td>
<td>2.68</td>
<td>62 (9)</td>
<td>46 (8)</td>
<td>45 (5)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>Medium (2.5–4.0 g)</td>
<td>2.55</td>
<td>59 (8)</td>
<td>47 (6)</td>
<td>51 (3)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Large (4.0–10 g)</td>
<td>2.70</td>
<td>24 (4)</td>
<td>23 (4)</td>
<td>27 (2)</td>
<td></td>
</tr>
<tr>
<td>BALB/cfC3H None</td>
<td>2.24</td>
<td>59 (6)</td>
<td>41 (5)</td>
<td>1 (6)</td>
<td>6 (6)</td>
</tr>
<tr>
<td>Medium (2.5–4.0 g)</td>
<td>2.93</td>
<td>74 (9)</td>
<td>42 (4)</td>
<td>57 (2)</td>
<td>2 (3)</td>
</tr>
</tbody>
</table>

a Final dilution, 1:800/ml; %, average inhibition of migration relative to control without mitogen or antigen; numbers in parentheses, number of mice tested.
b Final concentration, 1 µg/ml.
Cell-mediated Immune Responses to MTV Containing Mouse Milk and Purified MTV

In an investigation aimed at elucidation of the bases of reactivity to multiple antigens that may occur in MTV-positive tumors, migration studies were performed using PEC from BALB/cfC3H animals and for comparative purposes PEC from BALB/cCrgl. For migration inhibition with viral antigen we used MTV-containing Rill milk and as a "negative" control we used NIH Swiss milk, both kindly supplied by the Viral Cancer Program. Results of some such studies are presented in Chart 2. To our surprise there was significant inhibition of migration of PEC from BALB/cCrgl with the Rill milk. This was observed with normal as well as animals bearing the immunogenic MTV-negative D1-DMBA-3. Milk of Swiss mice did not inhibit migration appreciably. Strikingly different results were obtained with PEC from BALB/cfC3H mice. In most tests Rill milk did not cause inhibition of migration. Since these mice are known to have been exposed to MTV neonatally, the absence of inhibition implies some form of tolerance. It is, therefore, more remarkable that after transplantation with spontaneous mammary tumors the cells of these animals become responsive to MTV-positive milk.

A similar pattern was observed in the blastogenic transformation reaction. When BALB/cCrgl spleen lymphocytes (Chart 3) were tested with Rill milk, positive responses were observed in both normal and tumor-bearing animals. This response was increased in most experiments by the presence of D1-DMBA-3 tumors. This was apparently not due to viral antigens (as none have been detected by other methods including radioimmunoassay) but reflected on general hyperactivity of the host's lymphocytes caused by this tumor. When purified virus (obtained from the Virus Cancer Program from stocks at Meloy) was used as a stimulus, lymphocytes invariably responded with increased incorporation of [3H]thymidine with stimulation indexes from 2 to 6 in the case of normal animals and up to 13 in the case of animals bearing the MTV-negative D1-DMBA-3 tumor.

In contrast, lymphocytes from BALB/cfC3H mice showed no significant responses to MTV-positive milk or purified MTV unless they were first implanted with spontaneous tumor (Chart 4).

**Discussion**

The findings summarized in the present report justify several conclusions and form the basis for some hypotheses.

Although common antigens have been previously found to exist in mouse mammary tumors these were apparently virus antigens or virus-induced antigens. Common antigens appeared to be lacking in MTV-negative tumors originated by the carcinogenic action of DMBA.

A highly significant mark of distinction among DMBA tumors is the difference in immunogenic quality. Some are strongly immunogenic while others are devoid of any detectable immunogenic capacity. This was first noticed by Halpin et al. (7) by means of in vivo challenges. This distinction was confirmed in our laboratory by way of cell-mediated reactions, which were blastogenic transformation of lymphocytes and migration inhibition of PEC. These findings are
important in the context of human cancer where some patients fail to manifest immunological reactivity to their tumors. While weak responses may be attributable to a deficit in immune status, the failure may in fact reside in a deficiency in tumor-specific antigen.

Migration inhibition of PEC gave results that were in close agreement with the results obtained with the blastogenic transformation reaction. The correlation reflected the antigenic character of the tumor and the stage of disease of the host. Also, there were no cross-reactivities between the immunogenic and nonimmunogenic chemically induced tumors and between them and tumors of spontaneous origin. This degree of fidelity warrants more extensive application of these 2 tests in human breast cancer.

The lymphocytes of normal BALB/cCrGl mice did not react to antigens extracted from chemically induced tumors. However, they reacted to an antigen associated with MTV. This reactivity has been demonstrated by Blair et al.

The most crucial question is the relevance of these antigens and expressions of immunological activities to the origin and progression of mammary tumors. This question is assuming added importance in view of the demonstration of the horizontal transmission of mammalian C-type particles (9, 13). Regarding MTV, Blair (3) has provided some preliminary indications for horizontal transmission. Nevertheless, one must consider an alternative explanation, namely, that the reactivity of the lymphocytes is the result of sensitization to a viral protein of an endogenous origin. Bentvelzen (1) has reviewed several interesting aspects bearing on genetic transmission, repression, and induction of MTV and has proposed a germinal provirus hypothesis. More recently, Schlom et al. (14), using molecular hybridization techniques, have demonstrated in the BALB/c genome sequences corresponding to MTV-RNA. These sequences are apparently expressed by activation by such factors as aging and nonviral carcinogens. It is our hypothesis that the virus-associated antigens in BALB/cCrGl mice represent partial derepression of the genome. There is now substantial evidence for the occurrence of viral structural proteins and glycoproteins in cells free of virus. This was first demonstrated by Chen et al. (5) and Hanafusa et al. (8) in chicken embryo cells devoid of viral particles. More recently, it was determined that the cell surface antigen, G4x, which was originally thought to be a differentiation antigen of lymphoid cells (15), is also present in the envelope of murine leukemia virus (6, 16). What is important in the context of our work is that this antigen has been found on membranes of virus-free lymphocytes of strain 129 mice. The occurrence of a viral structural antigen on cells is usually regarded as an indication of infection or transformation. Since this antigen is found on cells in the absence of these 2 conditions its presence may denote an expression of a putative viral gene. The results in these 3 recent papers (6, 15, 16) provide strong support for the concept of selective (or partial) expression of viral genetic information invested in host chromosomes.

The question is concerned with the origin of sensitivity of lymphocytes in BALB/cCrGl mice. What antigen is responsible for the emergence of this state? One possible explanation invokes horizontal transmission. In our previous studies it was shown that this occurred with avian leukemia viruses. More recently, evidence has come forth for the horizontal transmission of mammalian C-type particles (9, 13). Regarding MTV, Blair (3) has provided some preliminary indications for horizontal transmission. Nevertheless, one must consider an alternative explanation, namely, that the reactivity of the lymphocytes is the result of sensitization to a viral protein of an endogenous origin. Bentvelzen (1) has reviewed several interesting aspects bearing on genetic transmission, repression, and induction of MTV and has proposed a germinal provirus hypothesis. More recently, Schlom et al. (14), using molecular hybridization techniques, have demonstrated in the BALB/c genome sequences corresponding to MTV-RNA. These sequences are apparently expressed by activation by such factors as aging and nonviral carcinogens. It is our hypothesis that the virus-associated antigens in BALB/cCrGl mice represent partial derepression of the genome. There is now substantial evidence for the occurrence of viral structural proteins and glycoproteins in cells free of virus. This was first demonstrated by Chen et al. (5) and Hanafusa et al. (8) in chicken embryo cells devoid of viral particles. More recently, it was determined that the cell surface antigen, G4x, which was originally thought to be a differentiation antigen of lymphoid cells (15), is also present in the envelope of murine leukemia virus (6, 16). What is important in the context of our work is that this antigen has been found on membranes of virus-free lymphocytes of strain 129 mice. The occurrence of a viral structural antigen on cells is usually regarded as an indication of infection or transformation. Since this antigen is found on cells in the absence of these 2 conditions its presence may denote an expression of a putative viral gene. The results in these 3 recent papers (6, 15, 16) provide strong support for the concept of selective (or partial) expression of viral genetic information invested in host chromosomes.

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The critical question is the relevance of these antigens and expressions of immunological activities to the origin and progression of mammary tumors. This question is assuming added importance in view of the demonstration...
of immunological reactivity in human patients to MTV antigens (2, 11) and therefore deserves extensive investigation.

Acknowledgments

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References


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