Circulating Immune Complexes in Rats Bearing 6-Thioguanine-resistant Variants of the 13762 Mammary Adenocarcinoma

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ABSTRACT

The relationship between immune complex (IC) formation and tumor cell metastatic potential was investigated in rats inoculated in the footpad with parental 13762 mammary adenocarcinoma cells or 6-thioguanine-resistant (TGR) variant cells. These cell lines are either highly metastatic (13762), nonmetastatic (TGR), or occasionally metastatic (TGRrev, TGRrevM). The 13762 TGR rat tumor model thus provides the opportunity to examine host immune responses to tumor cells of different phenotypes, but derived from the same parent tumor line. IC levels were low in 13762 tumor-bearing rats. In contrast, animals with TGR tumors had high levels of ICs in their sera, while animals bearing TGRrev and TGRrevM tumors had intermediate levels of ICs. In this rat tumor model system, IC formation is inversely related to the metastatic potential of the tumor lines.

INTRODUCTION

The relationship between tumor metastasis and the host's immune response is still not fully understood (3, 11). One aspect of the immune response during tumor metastasis that has received considerable attention is formation of circulating ICs (1, 5, 6, 14–16). Although circulating ICs are found in tumor-bearing hosts, levels vary with the type of neoplasm involved and with the clinical stage of the disease. This is not surprising, since tumors are heterogeneous (2) and formation of ICs is probably related to this tumor property.

There are few animal tumor models in which metastasis to lymph nodes and circulating ICs have been concurrently examined (5, 7, 8). Evaluating the relationship between IC formation and metastasis in these studies is difficult, because tumors of different types or origin or both were used. In our study (7), we found that IC formation varied in rats bearing different mammary adenocarcinomas. Although tumors of similar type were examined, no clear relationship between IC formation and metastasis was found. That the tumors used had different origins as well as being phenotypically heterogeneous may have led to the results that we obtained.

The only way to avoid complications posed by tumors of different origin would be to analyze IC formation in animals bearing clonally derived variants of the same tumor cell line. This approach became feasible with our recent isolation of TGR variants of the 13762 mammary adenocarcinoma (13). The 13762 TGR rat tumor model utilizes the highly metastatic 13762 parent cell line, the nonmetastatic TGR cell line, and the occasionally metastatic TGR revertant (TGRrev and TGRrevM) cell lines. With these cell lines, we were able to investigate IC formation in rats bearing tumor cells of different phenotypes (e.g., different metastatic potential), but derived from the same parent tumor line (e.g., same origin). A solid-phase porcine C1q enzyme immunoassay was used for IC detection. Circulating IC levels rose in all tumor-bearing rats as the primary tumor progressed. Interestingly, an inverse relationship was found between IC formation and the potential of the primary tumor cells to metastasize.

MATERIALS AND METHODS

Animals. Female Fischer F344 rats were used (Charles River Breeding Laboratories, Inc., Wilmington, MA). Serum samples were obtained prior to tumor inoculation and periodically thereafter. Serum preparation and storage were done as described previously (7).

Tumor Cell Lines. The 13762 MAT-B (ascites) cell line, which is syngeneic to F344 rats, was obtained from Dr. A. E. Bogden (Mason Research Institute, Worcester, MA). The 13762 mammary adenocarcinoma exhibits rapid primary tumor growth and extensive metastasis after inoculation of 10⁶ cells into a rat footpad (7).

Isolation of a TGR variant of the 13762 cell line has been described (13). In ep (<20 in vitro passages), this variant cell line proved to be nontumorigenic and nonmetastatic. However, in Ip (>30 in vitro passages), the cell line was tumorigenic but still nonmetastatic. Briefly, 5 × 10⁶ TGR(ep) cells were nontumorigenic in normal animals, while the same number of TGR(lp) cells consistently produced a primary footpad tumor. TGR(lp) cells were poorly tumorigenic following inoculation of 10⁶ cells.

Two tumorigenic and metastatic phenotype revertants were isolated from the TGR(ep) cell line and designated TGRrev and TGRrevM (13). Both revertants were more tumorigenic than were even TGR(lp) cells, since both produced primary tumors following inoculation of 10⁶ cells. Following inoculation of 5 × 10⁶ cells, both the TGRrev and TGRrevM cell lines showed frequent metastasis.

Table 1, Columns 2 and 3, summarizes the tumorigenic and metastatic phenotypes of the cell lines used.

Primary Tumor Growth-Metastasis. In all cases, 5 × 10⁶ cells were inoculated into the right hind footpad of an animal. In vitro growth of tumors, inoculation of cells, measurement of primary tumor growth, and detection of micro- or macrometastasis were all done as described previously (7). The 13762, TGR(ep), TGR(lp), TGRrev, and TGRrevM cells were used at passages 105, 12, 40, 15, and 15, respectively.

IC Detection. A solid-phase porcine C1q enzyme immunoassay was used to detect ICs (7). As before, heat-aggregated rat IgG was used as an IC-positive standard in all assays. For reasons given previously (7), we chose to express our IC data as increases in A₄₉₂ values for sera collected after tumor cell inoculation. For comparative purposes, we again give µg heat-aggregated IgG/ml equivalents for the maximum A₄₉₂ values observed.
Table 1
IC levels found in vivo and tumorigenic and metastatic characteristics of the 13762-TGR tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumorigenicity</th>
<th>Metastatic potential</th>
<th>Mean of maximum IC levels (A_492) above normal</th>
<th>General IC level</th>
</tr>
</thead>
<tbody>
<tr>
<td>13762</td>
<td>++++</td>
<td>++++</td>
<td>0.051 Low</td>
<td></td>
</tr>
<tr>
<td>TGR(ep)</td>
<td>–</td>
<td>–</td>
<td>0.409 Undetectable</td>
<td></td>
</tr>
<tr>
<td>TGR(lp)</td>
<td>+</td>
<td>–</td>
<td>0.182 Intermediate</td>
<td></td>
</tr>
<tr>
<td>TGRRev</td>
<td>++</td>
<td>+</td>
<td>0.190 Intermediate</td>
<td></td>
</tr>
<tr>
<td>TGRRevM</td>
<td>++</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Assessed by the number of cells required to consistently produce a primary rat footpad tumor (see "Materials and Methods" and Ref. 13). ++++, low number of cells needed; –, no tumor produced by a high number of cells.

**Assessed by ability of cells in a primary footpad tumor to metastasize to the regional draining lymph nodes and ultimately to the lungs (13). ++++, high; –, negative.

Mean of the maximum IC level of individual rats in each group. The Wilcoxon rank-sum test was used to compare the groups: 13762 versus TGR(lp), TGRRev, and TGRRevM, p < 0.01; TGR(lp) versus TGRRev and TGRRevM, p < 0.02; TGRRev versus TGRRevM, p > 0.2; and TGRRev or TGRRevM metastatic versus TGRRev or TGRRevM nonmetastatic, p > 0.2

Comparative ranking of general serum IC levels in rats bearing tumors initiated by the various cell lines.

ep, early passage (<20) in vitro; Ip, later passage (>30) in vitro.

RESULTS

13762 Tumor-bearing Animals. Following inoculation of 13762 tumor cells into a rat footpad, a primary tumor develops which invariably metastasizes to the draining regional lymph nodes and then to other parts of the body. Previously, we found low levels of ICs in rat inoculated with 10^6 13762 tumor cells (7). In this study, rats were inoculated with 5 x 10^6 13762 tumor cells so that comparisons could be made with rats given the same number of 13762-derived tumor cells.

Each of 7 rats inoculated with 5 x 10^6 13762 tumor cells developed a large primary footpad tumor by Day 28 (Chart 1B). A palpable (popliteal) lymph node metastasis, varying from 1 to 3 cm in diameter, was also detected in each animal at Day 28. As found previously (7), only low levels of ICs were detected in the 13762 tumor-bearing rats (Chart 1A), reaching a maximum mean A_492 of 0.052 on Day 21 (equivalent to approximately 11 μg heat-aggregated IgG/ml). IC levels then decreased slightly.

TGR Tumor-bearing Animals. Primary footpad tumors did not develop in 4 rats inoculated with 5 x 10^6 TGR(ep) cells. These animals were tested for circulating ICs over 6 to 7 weeks, and IC levels remained at the prebleed A_492 level of 0.070 ± 0.010 (S.E.). For example, at Day 18 post-tumor cell inoculation, the mean A_492 value found was 0.069 ± 0.007.

Each of 5 rats inoculated with 5 x 10^6 TGR(lp) tumor cells developed a primary footpad tumor with no evidence of regression (Chart 2B). These animals were sacrificed at Day 75 due to their large tumor burden. No palpable lymph node metastases were detected. Histological examination of the draining popliteal lymph node and nodes further up the lymphatic chain found no evidence of micrometastasis. In general, the draining lymph nodes of TGR(lp) tumor-bearing rats consisted of atrophied paracortical regions and remnants of a few germinal centers in marginal regions. Sinus histiocytosis was present in most of the lymph nodes.

Very high levels of circulating ICs were found in TGR(lp) tumor-bearing rats (Chart 2A). These reach a mean maximum A_492 of 0.306 on Day 44 (equivalent to approximately 57 μg heat-aggregated IgG/ml). A rapid increase in IC levels occurred between Day 20 and Day 44.

TGRRev and TGRRevM Tumor-bearing Animals. Each of 5 rats inoculated with 5 x 10^6 TGRRev cells and 4 rats inoculated with 5 x 10^6 TGRRevM cells developed a primary footpad tumor (Chart 3B). Three of the 5 TGRRev and 2 of the 4 TGRRevM tumor-bearing rats had palpable popliteal lymph node metastases (2 to 2.5 cm diameter) at the termination of the experiment.
Histological examination found no evidence of micrometastasis in the draining lymph nodes of the remaining TGRrev and TGRrevM tumor-bearing animals. These results fit with the earlier finding that approximately 50% of rats bearing TGRrev and TGRrevM tumors develop metastasis (13).

IC levels found in TGRrev and TGRrevM tumor-bearing rats (Chart 3A) were significantly less than those found in TGR(lp) tumor-bearing animals (Chart 2A) but higher than those found in 13762 tumor-bearing animals (Chart 1A). IC levels in TGRrev and TGRrevM tumor-bearing rats reached maximum mean \( A_{492} \) values of 0.125 and 0.148 (equivalent to approximately 28 and 32 \( \mu \)g heat-aggregated IgG/ml, respectively) on Day 20. Thereafter, the levels of circulating ICs tended to decrease. No differences were seen in IC levels of animals bearing metastatic TGRrev and TGRrevM tumors compared to those animals bearing nonmetastatic tumors.

Summary. Table 1, Columns 4 and 5, indicates the average of maximum \( A_{492} \) values for individual animals and the general IC levels in all animals inoculated with each of the cell lines used in this study.

DISCUSSION

One of the major questions asked in IC-cancer studies is whether circulating ICs can be used in predicting tumor progression in the host. In this study, we used an animal model and several tumor cell lines derived from the same origin to analyze the relationship between IC levels and metastatic potential of the tumor cells. General levels of circulating ICs were found to be inversely related to the metastatic potential of the tumor cell lines used (Table 1). This conclusion is based on the finding that rats bearing the poorly metastatic TGR developed high levels of ICs, while rats bearing the highly metastatic parent 13762 tumor developed only low levels of ICs. Rats bearing TGRrev and TGRrevM tumors, which metastasize approximately 50% of the time, developed intermediate IC levels.

In our earlier analysis of IC levels in rats bearing R3230AC tumors (7), animals bearing metastatic tumors had significantly lower IC levels than did animals bearing nonmetastatic tumors. This suggests that rats bearing metastatic TGRrev or TGRrevM tumors should have had lower IC levels than do rats bearing nonmetastatic tumors. Such was not the case, however. We believe that this difference is related to the phenotypic nature of the TGR revertant cells and the R3230AC cells. R3230AC cells, due to their long passage history, probably are phenotypically heterogeneous compared to the TGR revertant cells. Consequently, in R3230AC tumor-bearing rats the host's immune system interacts with a heterogeneous primary tumor, while in TGR revertant tumor-bearing rats the interaction is with a relatively homogeneous primary tumor. This means that IC formation in those animals experiencing tumor metastasis in these 2 instances need not necessarily follow a similar pattern.

Although it seems reasonable to expect that levels of circulating ICs would correlate with tumor burden, we did not find this with the 13762-TGR animal tumor model. A similar observation has been made in other animal tumor models (8, 9). Recent work, however, does provide an explanation for the inverse relationship that we found between IC levels and tumor metastasis. Earlier, we showed that the poor tumorigenicity of the TGR(ep) and TGR revertant tumor cells, as compared to the parent 13762 tumor cells, is due to an irradiation-sensitive rejection mechanism (13). This rejection mechanism is most probably immunological. More recently, we found that TGR(ep and lp) cells are more immunogenic than are 13762 cells (demonstrated by cell-mediated cytotoxicity studies*). Also, TGRrev and TGRrevM cells were found to be more immunogenic than were 13762 cells but less immunogenic than were TGR(ep and lp) cells. In our model system, it appears that the more immunogenic a tumor cell is, the more likely is metastasis to be prevented by immunological mechanisms, one parameter of which is IC formation. In other words, IC formation in the 13762-TGR animal tumor model appears to be directly related, while metastasis is inversely related to the immunogenicity of the various tumor cell lines.

The lack of a detectable increase in ICs in animals inoculated with TGR(ep) cells deserves comment here. It is obvious that a phenotypic change has occurred in the TGR cells between in vitro passages 20 and 30 (Table 1). Although the nature of this change is unknown, it is probably related to immunogenicity of the cells, as already briefly discussed. This means TGR(ep) cells are sufficiently immunogenic so that even large numbers (i.e., 5 \( \times 10^6 \)) of cells are efficiently rejected. That elevated IC levels were not found at the first sampling time of 7 days post-cell inoculation probably means that most of the injected tumor cells have been cleared by this time. It also indicates strong primary tumor growth is needed before elevated levels of circulating ICs can be consistently detected in animals bearing one of the 13762 TGR tumors. Consequently, we believe that the lack of increased IC formation in rats inoculated with TGR(ep) cells in no way detracts from our conclusion that IC levels and metastatic potential of the 13762 TGR tumor cells are inversely related.

ICs can arise in tumor-bearing animals in one of 2 ways. They may be in the form of tumor antigen-antibody complexes or idiotype-antiidiotype complexes (12, 17). Formation of the former

* D. B. S. Hoon and I. Ramshaw, unpublished results.
type of IC will depend upon various factors including immunogenicity of the tumor antigens and rate at which these antigens shed. For favorable formation of tumor-antibody complexes, the rate of antibody synthesis and tumor antigen shedding must be in equilibrium. Any change in either of these factors could lead to altered levels of ICs. Alternatively, a portion or all of the ICs found in 13762-TGR tumor-bearing rats could be idiotype-anti-idiotype complexes. Such ICs are immunoregulatory, since they usually function to modulate specific antibody synthesis in an animal (4). Levels of such complexes can be expected to increase until immunoregulatory mechanisms are induced and feedback effects come into play.

From data presented in Charts 1A, 2A, and 3A, it is evident that IC levels do vary in animals bearing tumors caused by one of the 13762-TGR tumor cell lines. In animals bearing tumors with metastatic potential (13762, TGRrev, and TGRrevM), IC levels plateaued or started to decrease about 3 weeks post-tumor cell inoculation. In contrast, in animals bearing nonmetastatic TGR(p) tumors, IC levels increased rapidly between 3 and 6 weeks post-tumor cell inoculation and only then began to decline. These changes in IC levels must be the net result of temporal alterations in tumor cell-host immune interactions and immune regulation. Whether ICs detected at any given time are primarily antibody-complexed tumor antigens or idiotype-antidiotype complexes remains to be determined. Finally, the results presented document the benefit associated with using tumor cell lines derived as clonal variants from a parent tumor cell line, rather than working with similar but heterogeneous tumors from different origins. Such an approach readily allows specific aspects of the tumor-host relationship to be focused upon. Recently, other investigators have also shown the advantage of using a parent variant tumor cell model for comparing the host's immune responses to tumor cells varying in phenotypic expression (10, 18). Clearly, exploring specific aspects of the tumor-host relationship by using tumors clonally derived from the same origin can be expected to give better insight of what transpires during metastasis of naturally occurring, heterogeneous tumors.

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