Differences in the Sensitivities of Murine Metastatic Lymphoma/ Lymphosarcoma Variants to Macrophage-mediated Cytolysis and/or Cytostasis

Karen M. Miner and Garth L. Nicolson

Department of Tumor Biology, University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030

ABSTRACT

A metastasizing animal tumor model for large cell lymphoma or lymphosarcoma has been established by sequential selection in vivo of the RAW117 parental cell line in BALB/c mice for enhanced colonization of liver or lung and in vitro for lack of binding to immobilized lectins. The parental RAW117 and selected sublines and clones derived from these were compared for their sensitivities in vitro to polyinosinic:polycytidylic acid-activated syngeneic macrophages in cytolysis and cytostasis assays. Activated but not unactivated macrophages had differing effects on RAW117 sublines and clones. The least metastatic (parental) cell line was the most sensitive to activated macrophage-mediated cytolysis and cytostasis, while the most metastatic subline was the least sensitive in these assays. Intermediate metastatic sublines or clones were usually less sensitive in one or both of the assays. These antitumor activities were negligible at 24 hr in the continuous presence of polyinosinic:polycytidylic acid but were clearly apparent by 48 to 72 hr. The results suggest that differential sensitivity to host macrophage surveillance mechanisms can occur in malignant cell subpopulations.

INTRODUCTION

Metastasis involves a complex series of highly selective sequential steps resulting in the survival and growth of a small fraction of the malignant cells to form secondary tumors (5, 9, 17, 20). This suggests that highly metastatic cells possess unique properties that aid in the establishment of metastatic foci (9, 17, 20). In order to elucidate the tumor cell properties important in metastatic events, experimental animal models have been developed that consist of cell sublines derived from the same parental tumor cell population but possessing varying metastatic capacities (9, 17). We have developed such a model for murine large cell lymphoma or lymphosarcoma based on the recently established cell line RAW117. This tumor is similar to human large cell lymphoma or lymphosarcoma in its cell lineage, cell and tumor structure, and organs colonized (2). Parental RAW117 cells are capable of forming some solid tumor nodules in liver, spleen, lung, and lymph nodes of BALB/c mice after injection of 5 x 10^3 viable tumor cells s.c. (22) or i.v. (2).

The parental RAW117 cell line has been used to sequentially select cell sublines that have enhanced liver (2) or lung colonization properties or, alternatively, to select cell sublines for modifications in cell surface properties, such as loss of lectin-binding sites (21). These selected, highly metastatic RAW117 cells show alterations in their cell surface proteins (19), glycoproteins (16, 21, 22), antigens (18, 22), partitioning behavior in 2-phase polymer solutions (16), and survival in normal and immune-impaired mice (23). In the latter series of experiments, we found that low metastatic RAW117 sublines possessed much higher metastatic potentials in mice with impaired macrophage function but not in mice with impaired T-cell or NK cell immune systems. However, in highly metastatic RAW117 sublines, there was no significant effect on metastatic properties when host immune systems were suppressed (23). Using in vitro-activated macrophages (15, 24, 26), we show here that metastatic RAW117 sublines and clones are differentially sensitive to macrophage-mediated cytolysis and/or cytostasis.

MATERIALS AND METHODS

Animals. Inbred 5- to 7-week-old female BALB/c mice were obtained from the virus-free colony of Charles River Breeding Laboratories, or the NCI-Frederick Cancer Research Facility (Frederick, Md.) and were maintained under guidelines established by the University of Texas System Cancer Center and the National Research Council. Animals were routinely quarantined for 2 weeks, and their weights were recorded twice weekly. Routine assays included screening for microorganisms and murine viruses. Animals were fed normal rodent chow and unchlorinated spring water ad libitum.

Cell Lines and Culture Conditions. RAW117 cells were grown as suspension cultures in DME supplemented with 10% FBS (Flow Laboratories, Inc., McLean, Va.) and 1% nonessential amino acids without antibiotics. Cell cultures were kept at 37° in a humidified incubator with 5% CO2:95% air.

The methods for sequential in vivo selection of RAW117 sublines with enhanced abilities to form solid tumors in the livers of BALB/c mice and procedures for in vitro selection of lectin-binding variants are reported elsewhere (2, 21). Parental RAW117 cells and derivative sublines were sequentially (twice) subjected to cell cloning using limited dilution methods (22). All cells were used within 10 passages from frozen stocks to eliminate problems of phenotypic drift in metastatic properties (17, 18).

Cell clones in this system were tested for metastatic properties at the same passage numbers as the cytolysis and cytostasis assays.

Experimental Metastasis Assays. RAW117 sublines or clones were assayed for organ colonization (experimental metastasis) after i.v. injection of 5 x 10^6 viable tumor cells/0.2-ml inoculum (2). Experiments were terminated at approximately 10 to 12 days after injection, whereupon visible tumor nodules were counted in all major organs. All organ colonization assays were confirmed by histology.

Cytolysis and Cytostasis Assays. Peritoneal exudate cells were obtained by injecting 3 ml of thioglycollate (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.) i.p. into BALB/c mice.
Approximately 15 to 20 × 10⁶ peritoneal exudate cells/mouse were harvested 3 days later by aseptic peritoneal cavity lavage with 7 to 8 ml of cold Dulbecco’s phosphate-buffered saline, pH 7.2. The exudate was pooled and centrifuged (400 × g) for 5 min at 4°C, and the cell pellet was resuspended in DME containing 10% FBS and plated in 1.0-ml aliquots in wells of a 24-well plate (Linbro Scientific, Inc., Hamden, Conn.; Flow Laboratories) at different cell densities. After 4 hr of incubation at 37°C, the macrophage monolayers were washed once with DME:10% FBS to remove nonadherent cells. Esterase staining indicates that this population contains at least 95 to 98% of esterase-positive cells (24). One-half of the wells in each plate were treated with poly I:C (40 μg/ml; P-L Biochemicals, Inc., Milwaukee, Wis.) in DME:0.1% lactalbumin hydrolysate and the remaining one-half of the wells in the plate received DME:0.1% lactalbumin hydrolysate without poly I:C. After activation, the macrophage monolayers and controls were maintained by s.c. injection of RAW117 cells (22). The results show that the parental RAW117-P line is of very low metastatic potential (median number of liver tumors, 1; range, 0 to 10) clearly that the parental RAW117-P line is of very low metastatic potential (median number of liver tumors, 1; range, 0 to 10) and that the parental RAW117-P line is of very low metastatic potential (median number of liver tumors, 1; range, 0 to 10) and another subline in these assays (Table 1).

**RESULTS**

The biological properties of the parental RAW117 line and various selected sublines, as well as those of certain cell clones obtained from these, are listed in Table 1. In these experimental metastasis assays, tumor cells were injected i.v. into groups of 10 BALB/c mice, and the numbers of visible tumor colonies were determined after 12 to 14 days. Animal died with >200 liver tumors before assay date.

**Statistical Analyses.** Differences among control and experimental assays were analyzed for statistical significance by one-way analysis of variance. The statistical significance of differences between sublines using 6 dependent observations of percentage of cytolysis or cytostasis was determined by the Mann-Whitney U test.

---

### Table 1

**Biological properties of RAW117 sublines and clones**

<table>
<thead>
<tr>
<th>Subline or clone</th>
<th>Selection</th>
<th>No. of gross liver tumor colonies</th>
<th>Liver</th>
<th>Lung</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW117-P</td>
<td>None</td>
<td>0, 0, 0, 0, 0, 1, 2, 2, 4</td>
<td>4/10</td>
<td>1/10</td>
<td>2/10</td>
</tr>
<tr>
<td>RAW117-P Cl. 21</td>
<td>None</td>
<td>0, 0, 0, 1, 10, 12, 29, 42, 59, 200</td>
<td>5/10</td>
<td>1/10</td>
<td>2/10</td>
</tr>
<tr>
<td>RAW117-P Con A&lt;sup&gt;1&lt;/sup&gt; Cl. 40</td>
<td>10×-immobilized Con A</td>
<td>21, 31, 42, 59, 100, 200, 200, 200, &gt;200&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10/10</td>
<td>1/10</td>
<td>6/10</td>
</tr>
<tr>
<td>RAW117-L17</td>
<td>17× lung colonization</td>
<td>0, 0, 0, 0, 2, 9, 14, 22, 26, 28</td>
<td>6/10</td>
<td>8/10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2/10</td>
</tr>
<tr>
<td>RAW117-H10 WGA&lt;sup&gt;2&lt;/sup&gt; Cl. 15</td>
<td>10× liver colonization and 10×-immobilized WGA</td>
<td>0, 3, 4, 4, 15, 29, 40, 46, 48, 50</td>
<td>9/10</td>
<td>1/10</td>
<td>2/10</td>
</tr>
<tr>
<td>RAW117-H10</td>
<td>10× liver colonization</td>
<td>&gt;200&lt;sup&gt;b&lt;/sup&gt;, &gt;200&lt;sup&gt;b&lt;/sup&gt;, &gt;200&lt;sup&gt;b&lt;/sup&gt;, &gt;200&lt;sup&gt;b&lt;/sup&gt;, &gt;200&lt;sup&gt;b&lt;/sup&gt;, &gt;200&lt;sup&gt;b&lt;/sup&gt;, &gt;200&lt;sup&gt;b&lt;/sup&gt;, &gt;200&lt;sup&gt;b&lt;/sup&gt;, &gt;200&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10/10</td>
<td>2/10</td>
<td>10/10</td>
</tr>
<tr>
<td>RAW117-H10 Cl. 3</td>
<td>10× liver colonization</td>
<td>10, 14, 22, 56, 63, 100, 101, 110, 150, 200</td>
<td>10/10</td>
<td>2/10</td>
<td>5/10</td>
</tr>
</tbody>
</table>

---

<sup>a</sup> Selections performed as described in the references.<br>
<sup>b</sup> Viable RAW117 cells (5 × 10⁵ cells/0.2-ml inoculum) were injected i.v. into groups of 10 BALB/c mice, and the numbers of visible tumor colonies were determined after 12 to 14 days.<br>
<sup>c</sup> Animal died with >200 liver tumor colonies before assay date.<br>
<sup>d</sup> Median; 20, range 0 to 42.

---

<sup>a</sup> Unpublished observations, K. W. Brunson, C. L. Reading and G. L. Nicolson.

---

is the number of target cells in wells without macrophages.
insignificant in the presence of activated macrophages (Table 2).

These results were quite reproducible. Differences in cytolyis and cytostasis between low-malignant RAW117-P cells and high-malignant RAW117-H10 cells were significant at the 0.002 and 0.001 levels, respectively, by the Mann-Whitney U test.

The RAW117 sublines and clones of metastatic potential intermediate to the low-malignant RAW117-P and high-malignant RAW117-H10 subline usually yielded differing results in the cytolyis or cytostasis assays. For example, RAW117-H10 WGA10 Cl.15 (selected in vivo for liver colonization, in vitro for loss of adherence to immobilized wheat germ agglutinin, and then cloned as in Ref. 22) was relatively sensitive to macrophage-mediated cytolyis but was as insensitive in growth inhibition assays as was the H10 subline (Table 2). Similarly, RAW117-P Con A Ch 0 Con A Cl.40 (selected in vitro from the parental line for loss of adherence to immobilize concanavalin A and then cloned as in Ref. 22) was killed well in the cytolyis assay but was consider-
ably less sensitive than were parental cells in the cytostasis assay (Table 2).

The results expressed in Table 2 were not due to differential binding of the suspension-grown target cells to the adherent macrophage cultures. This was demonstrated in the following control experiments. (a) The macrophage-bound RAW117 cells at the end of the 72-hr assays were removed by brief (2 min) treatment with a 0.1% trypsin solution. This concentration of trypsin removes essentially all of the macrophage-bound RAW117 cells but does not remove adherent, phagocytic cells from macrophage cultures even after 20 min of such treatment. For example, trypsin treatment removed approximately 1% of the total RAW117-P or -H10 cells. Obviously, these low numbers of released tumor cells do not change the results shown in Table 2, and the low numbers of target cells bound to the adherent macrophages after 72 hr indicate that differential binding of low- and high-malignant RAW117 cells to macrophages does not occur. (b) A brief pretreatment of adherent macrophages with a

### Table 2

| Target subline or clone | Effector: target ratio | Av. no. of target cells (10^4) | % of cytolysis | Significance* | Av. no. of target cells (x 10^4) | % of cytostasis | Significance*
|------------------------|------------------------|-----------------|--------------|-------------|-----------------|--------------|-------------
| RAW117-P               | None                   | 4,810 ± 330     | 40           | 0.001g      | 104,360 ± 7,360 | 24           | 0.001       |
|                        | 5:1                    | 2,870 ± 76      | 60           |             | 80,590 ± 1,110  | 52           |             |
|                        | 10:1                   | 1,930 ± 78      | 65           |             | 52,440 ± 1,980  | 78           |             |
|                        | 25:1                   | 1,700 ± 48      |              |             | 26,480 ± 7,830  |              |             |
| RAW117-P Cl. 21        | None                   | 4,870 ± 620     | 56           | 0.001g      | 96,730 ± 880    | 23           | 0.005       |
|                        | 5:1                    | 2,140 ± 250     | 61           |             | 72,880 ± 3,620  | 32           |             |
|                        | 10:1                   | 1,920 ± 315     |              |             | 65,740 ± 1,850  | 54           |             |
|                        | 25:1                   | 1,820 ± 500     |              |             | 45,570 ± 1,070  |              |             |
| RAW117-P Con A Ch 40 Cl. 40 | None | 4,830 ± 520     | 35           | 0.001       | 86,130 ± 2,220  | 8            | 0.01        |
|                        | 5:1                    | 3,150 ± 310     |              |             | 93,110 ± 2,200  |             |             |
|                        | 10:1                   | 2,670 ± 350     |              |             | 81,950 ± 680    | 8            |             |
|                        | 25:1                   | 1,920 ± 180     |              |             | 66,700 ± 4,760  | 22           |             |
| RAW117-L17             | None                   | 2,540 ± 120     | 58           | 0.001       | 76,650 ± 2,820  | 63           | 0.001       |
|                        | 5:1                    | 1,070 ± 110     |              |             | 36,720 ± 2,300  | 66           |             |
|                        | 10:1                   | 830 ± 120       |              |             | 29,200 ± 250    | 66           |             |
|                        | 25:1                   | 960 ± 290       |              |             | 15,110 ± 3,120  | 86           |             |
| RAW117-H10 WGA10 Cl. 15 | None | 3,210 ± 290     | 34           | 0.001       | 88,800 ± 2,870  | 15           | NS^h        |
|                        | 5:1                    | 2,120 ± 170     |              |             | 101,200 ± 2,040 |             |             |
|                        | 10:1                   | 814 ± 120       |              |             | 99,730 ± 4,730  | 13           |             |
|                        | 25:1                   | 1,570 ± 190     |              |             | 87,540 ± 2,870  |             |             |
| RAW117-H10             | None                   | 5,560 ± 720     | 14           | 0.025g      | 99,540 ± 5,280  | 7            | NS^h        |
|                        | 5:1                    | 4,760 ± 170     |              |             | 105,390 ± 2,730 |             |             |
|                        | 10:1                   | 3,900 ± 410     |              |             | 103,850 ± 1,550 |             |             |
|                        | 25:1                   | 3,750 ± 290     |              |             | 97,580 ± 4,060  | 2            |             |
| RAW117-H10 Cl. 3       | None                   | 5,570 ± 880     | 19           | 0.025g      | 102,670 ± 2,190 | 4            | NS^h        |
|                        | 5:1                    | 4,500 ± 220     |              |             | 106,670 ± 4,410 |             |             |
|                        | 10:1                   | 4,070 ± 460     |              |             | 102,030 ± 2,600 |             |             |
|                        | 25:1                   | 2,980 ± 450     |              |             | 86,710 ± 2,720  |             |             |

* Effector cells were thioglycolate-stimulated BALB/c mouse peritoneal macrophages activated in vitro in the continuous presence of poly I:C. 40 µg/ml. None, no effector cells.

^ Target cells (5 x 10^4) were treated with mitomycin C (5 µg/10^6 cells) and plated in the presence or absence of macrophages.

# Percentage of cytolysis was calculated as % of cytolysis = (C - E) x 100

where E and C are the remaining number of target cells in wells with or without macrophages, respectively.

# Significance between control and experimental samples at the indicated level was determined by one-way analysis of variance.

## Viable target cells (2 x 10^4) were plated in the presence or absence of macrophages.

# Percentage of cytostasis was calculated as percentage of cytostasis = 1 - (C - E) x 100, where E and C are the remaining number of target cells in wells with or without macrophages, respectively, and T is the number of target cells added to each well.

# Significant difference (p < 0.002) between subline RAW117-H10 and RAW117-P or RAW117-P Cl.21 was determined by the Mann-Whitney U test.

# NS, not significant by one-way analysis of variance.
low concentration of formaldehyde (0.5% for 3 min at room temperature) under conditions which preserve cell surface determinants (25) results in complete loss of cytolysis and cytostasis activities, indicating that viable, adherent macrophages are essential for obtaining the results in Table 2. The same low levels (<1%) of RAW117 cells were bound to the formalin-fixed macrophages after 72 hr.

That our cytolysis and cytostasis assays measured macrophage-mediated processes and not adherent natural cell cytolysis or cytostasis was demonstrated in control experiments in which poly(I:C) activation was eliminated. In general, the results with unactivated macrophages indicated that percentage of natural cytolysis is low (usually below 6% at effector:target ratios of 10:1) and not reproducible. In order to further rule out natural cell cytolysis, we performed the assay at 24, 48, and 72 hr in the continuous presence of poly(I:C) (Table 3). The results indicate that the cytolysis of RAW117 cells takes 48 to 72 hr to reach maximum levels and is barely detectable at 24 hr. At 48 or 72 hr, there are clearly differences in the cytolysis between the parental RAW117-P and RAW117-H10 cells (Table 3). The slower rate of increase in cytolysis with the H10 cells suggests that some of these cells might escape macrophage killing, since the doubling times of all clones and sublines are similar (11 to 12 hr) (23).

DISCUSSION

Data from different tumor-host systems indicate that cell-mediated antitumor immune systems can enhance, inhibit, or have no effect on tumor growth and/or metastasis (7, 8, 9, 17). Of the immune mechanisms that have been examined and found effective in killing certain metastasizing tumor cells, the most promising are mediated by T-cells, NK cells, or macrophages. Unfortunately, the most metastatic cells in tumors are often more resistant to T-cell- or NK cell-mediated host immunity (11-13). Activated macrophage antitumor responses are known to be effective in eradicating melanoma cells growing at metastatic sites, and it has been suggested that macrophages may act similarly against all tumor cells in local or metastatic lesions (4, 6, 10). However, tumors of lymphoid origin were not examined in these studies.

We have used poly(I:C)-activated adherent syngeneic peritoneal macrophage cultures as the effector cell population (15, 24, 26). These cultures do not appear to contain significant numbers of NK or NC cells by the following criteria: (a) the effector cells are adherent and are greater than 95% morphologically, phagocytically, and enzymatically (esterase positive), typical of macrophages but not of NK or NC cells (14, 24); (b) in the absence of poly(I:C) activation, there is little cytolysis or cytostasis activity (<5% in the 72-hr assays), and what is present is inconsistent from one experiment to another (15, 24); (c) these cultures do not show the high rates of killing of the usual NK targets, such as YAC-1 (24, 26); (d) the kinetics of poly(I:C) activation and the appearance of significant activity (48 to 72 hr) are more consistent with macrophage than with NK- or NC-mediated mechanisms (24); and (e) similar macrophage cultures can be activated by macrophage activation factor or muramyl dipeptide (24, 26). Collectively, these data indicate that macrophages rather than NK or NC cells are mediating the cytolysis and cytostasis activities.

Further support for the conclusion that macrophages are mediating the effects shown here has come from in vivo experiments in which antitumor immune functions have been impaired by a variety of methods (23). Although impairment of antitumor responses by using 400 R 60Co-irradiated or variously aged BALB/c nude (nu/nu) mice was not effective in modifying the metastatic properties of any of the RAW117 cell lines or clones, inhibiting macrophage function by placing chlorine in the drinking water or giving animals injections of trypan blue, silica particles, carrageenan, cyclophosphamide, or pristane dramatically enhanced the malignant properties of parental cells but had little or no effect on highly metastatic RAW117 cells in vivo (23).

We have found that highly metastatic RAW117 lymphoid tumor cells are less sensitive to activated macrophage-mediated cytolysis and cytostasis, suggesting that macrophages or their secreted products can distinguish between malignant subpopulations in certain tumors. Since no difference in binding of the RAW117 cells to macrophages was found, cytolytic or cytostatic substances released from such activated macrophages (15, 24) could act differently on various malignant cell subpopulations. Our results also suggest that cell subpopulations with differing malignant properties in other tumor systems may also be differ-
Differential Macrophage Sensitivities of Murine Lymphoma-Lymphosarcoma Variants

entially sensitive to activated macrophages or their secreted products, an observation we have made recently with highly metastatic brain-colonizing and lung-colonizing B16 melanoma cells (15). Differential sensitivity to activated macrophage cytolysis and/or cytostasis may be one, but certainly not the only, important characteristic allowing some malignant tumor cells to survive and proliferate at metastatic sites.

ACKNOWLEDGMENTS

We thank J. Klostergaard, G. A. Granger, and P. Williams for assistance and advice.

REFERENCES


Differences in the Sensitivities of Murine Metastatic Lymphoma/Lymphosarcoma Variants to Macrophage-mediated Cytolysis and/or Cytostasis

Karen M. Miner and Garth L. Nicolson


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/43/5/2063

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.