Macrophage Content of Spontaneous Metastases at Different Stages of Growth¹

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ABSTRACT

The macrophage content of spontaneous metastases has been quantified morphometrically for a panel of rodent tumors at different stages of metastatic tumor growth. Using a histochemical technique to selectively stain macrophages, we have evaluated the relative content of macrophages in spontaneous pulmonary metastases from the 13762NF MTLn3 rat mammary adenocarcinoma and the B16-BL6 mouse melanoma, as well as in spontaneous hepatic metastases from the M5076 mouse reticulum cell sarcoma and from autochthonous reticulum cell sarcomas in SJL/J mice. Between 112 and 254 separate, individual metastases were evaluated for each of these tumors. The data show that the relative macrophage content of very small metastases is high. However, as metastases grow the relative macrophage content falls, reaching uniformly low levels by the time the metastases are 0.5 mm in diameter. These data are very similar to our previous observations on experimental metastases, where the same pattern of high macrophage content in small metastases was seen. Finding the same pattern in more slowly growing, spontaneous metastases of tumors derived from several different tissues and in two species suggests that the fall in relative macrophage content is not a phenomenon isolated to experimental metastases, a particular site, or a tissue of origin for the tumor. The relative decrease in macrophage content may thus be a general phenomenon with important implications for immunotherapy directed to enhancing the tumoricidal activity of macrophages.

INTRODUCTION

Macrophages are believed to be important effector cells in host defense against neoplasms (1), and a variety of agents that activate macrophage tumoricidal activities have been reported to enhance host resistance to tumors or to promote destruction of established tumors (2-5). However, therapy designed to activate macrophages to the tumoricidal state is only effective in eradicating minimal tumor burdens (2, 5). The reasons for failure of these therapies to eradicate larger tumor burdens are poorly understood. In contrast to chemotherapy and specific immunotherapy which result in the selection and rapid emergence of resistant tumor cell subpopulations, the evolution of tumor cell variants which are resistant to macrophage-mediated cytolysis is uncommon (1, 2, 6, 7), though tumor cells do show graded susceptibility to macrophage-mediated cytolysis and/or cytostasis (8-12). Another explanation for the failure of non-specific immunotherapy with macrophage-activating agents to eradicate larger, established metastases is the inability of host defense mechanisms to keep pace with tumor growth. Although the macrophage content of large subcutaneous tumors (13) and metastases (14) is relatively constant, we have shown that the macrophage content of experimental micrometastases produced by i.v. inoculation of B16 murine melanoma cells is high in small, "early" metastases, but falls rapidly as metastases grow, remaining at uniformly low levels in lesions containing more than 1000 tumor cells (15). The purpose of the current experiments was to determine if a similar decrease in density of intratumoral macrophages was also characteristic of the progressive growth of more slowly growing metastases arising spontaneously from autochthonous or transplanted primary tumors or was merely a peculiarity of the rapid tumor growth occurring in organs after colonization by direct i.v. inoculation of tumor cells. In this paper, we report the analysis of the macrophage content of murine lung metastases produced by the dissemination of B16 cells implanted in the foot pad, 13762NF MTLn3 rat mammary adenocarcinoma implanted in the mammary fat pad, and hepatic metastases in mice bearing spontaneous or transplanted reticulum cell sarcomas. The results obtained with these three histotypically diverse neoplasms indicate the macrophage content of spontaneous metastases decreases rapidly in a similar fashion to experimental metastases in these species.

MATERIALS AND METHODS

Animals. Eight- to 12-wk-old female C57BL/6J mice were obtained from the Department of Laboratory Animal Sciences of Smith Kline & French Laboratories. Eight-mo-old retired breeding female SJL/J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Eight-wk-old female Fischer 344/CDL rats were obtained from Charles River Breeding Laboratories (Kingston, NY). Animals were allowed free access to pelleted food and water and were maintained under the guide lines defined in the "Guide for the Care and Use of Laboratory Animals" by the Institute of Laboratory Animal Resources, National Research Council.

Tumors and In Situ Labeling of Tumor-associated Macrophages. B16-BL6 murine melanoma cells were maintained in vitro as monolayers in Dulbecco's modified Eagle's medium with l-glutamine and D-glucose (1000 mg/liter) and sodium pyruvate, supplemented with 10% fetal bovine serum (Grand Island Biological Co., Grand Island, NY) as described (5). Single cell suspensions were obtained by treating monolayers with 0.25% trypsin and 0.01% EDTA for 1 min (Grand Island Biological Co.).

Primary tumors were produced by injection of 5 x 10⁶ viable B16-BL6 cells into the rear foot pad as described (5). Forty-six days after injection of tumor cells, when the foot pad tumors were approximately 1 cm in diameter, mice were given injections i.v. of 0.2 ml of iron dextran (Sigma Chemical Co., St. Louis, MO) diluted 50:1 with saline, to label tumor-associated macrophages for histochemical staining (see below). We have shown previously that iron dextran reliably labels phagocytic macrophages in tumors and micrometastases (15, 16). Mice were sacrificed 24 h after the injection of iron dextran, and their lungs were removed and fixed in phosphate-buffered formalin for subsequent morphometric and histological studies.

M5076 murine reticulum cell sarcoma cells (17), kindly provided by Dr. Randall Johnson, Smith Kline & French Laboratories, were maintained by serial i.p. passage in C57BL/6J mice. Primary tumors were produced by injection of 3 x 10⁸ cells into the right inguinal fat pad of five C57BL/6J mice. Twenty-one days after transplant, mice were given injections of iron dextran to label tumor-associated macrophages as described above. Liver tissue was removed and fixed in phosphate-buffered formalin.

Liver metastases were also evaluated in SJL/J mice bearing autochthonous reticulum cell sarcomas. Seventy to 80% of female mice of this strain develop neoplasms between 8 and 12 mo of age (18). Four 14-mo-old female SJL/J mice bearing palpable lymph nodes were given...
injections of iron dextran to label tumor-associated macrophages as described above. Histological examination revealed that all mice had multiple liver metastases.

Cells of the cloned subline (MTLn3) of the 13762NF rat mammary adenocarcinoma were maintained in vitro in α-modified Eagle’s medium supplemented with 10% fetal bovine serum as described (19). Single cell suspensions were obtained by treating monolayers with 0.25% trypsin in calcium-magnesium-free Dulbecco’s phosphate-buffered saline (Grand Island Biological Co.). Primary tumors were produced by injection of 1 x 10⁶ viable cells into the mammary fat pad of 5 rats anesthetized with methoxyflurane as described (19). Twenty-nine days after injection of tumor cells, rats were given injections i.v. of 0.1 ml of undiluted iron dextran to label tumor-associated macrophages. Rats were killed 24 h later, and their lungs were removed and fixed in phosphate-buffered formalin.

Quantification of Tumor-associated Macrophages. Formalin-fixed tissues containing metastases were dehydrated and embedded in paraffin by routine methods. Paraffin sections were stained to identify macrophages that had phagocytosed iron dextran using a two-step histochemical staining procedure described previously (15). Briefly, sections were treated with acidic potassium ferrocyanide to convert inorganic ferric iron to Prussian blue. The catalytic activity of Prussian blue was then used to selectively stain macrophages with modified diaminobenzidine medium which contained 10 times the normal concentration of H₂O₂ (0.1%). Sections were counterstained with hematoxylin or methyl green. The macrophages stain brown, while their nuclei and those of tumor cells stain blue or green. Tissue sections containing metastases of the B16 melanoma were bleached with 10% H₂O₂ in methanol for 24 h between the ferrocyanide and diaminobenzidine staining steps to remove melanin pigment.

Morphometric Analysis. The number of macrophages within individual metastases was measured directly from tissue sections using a Zeiss Videoplan (Carl Zeiss, Inc., Thornwood, NY) and a microscope equipped with a drawing tube. The number of stained cells in individual metastases was counted, and the surface area of the metastasis and the number of macrophages per unit area (number/mm²) were determined using the commercially available software programs for the Videoplan as described (15). Scatter diagrams, regression analyses, and tests of significance (F test) were performed with commercial software distributed by the manufacturer for the Videoplan.

**RESULTS**

**Macrophage Content of Lung Metastases.** The distribution of macrophages in spontaneous lung metastases of different sizes arising from 13762NF MTLn3 mammary tumor cells implanted in the foot mammary fat pad is shown in Figs. 1 and 2. Fig. 1 shows two small metastases. The number of macrophages is low, but the relative number of macrophages to tumor cells is high. In contrast, the typical pattern found in larger metastasis is illustrated in Fig. 2. The lesion contains a similar number of macrophages than the smaller metastases in Fig. 1, but the density of macrophages is much lower. Similar results were obtained with B16-BL6 metastases. To quantify the decrease in the relative number of macrophages in metastases, the number of macrophages per unit area was measured. Figs. 3 and 4 show the data for 13762NF MTLn3 and B16-BL6 metastases, respectively. In the top of each figure, the density of macrophages present within sections of single metastases is plotted against the sectional area of the same lesions. Each point represents the measurement from a separate, individual metastasis. In lung metastases produced by B16-BL6 or 13762NF MTLn3 cells, the density of macrophages falls rapidly with progressive enlargement of the metastatic lesions and plateaus at uniformly low levels. These results indicate that our previous observations of this phenomenon, using lung tumor colonies produced by i.v. injection of tumor cells, did not reflect events peculiar to a particular species, cell lineage, or inoculation method. In Figs. 3 and 4, bottom, the same data are plotted as the logarithm of macrophage density versus the logarithm of the cross-sectional area of different individual metastases. Linear regression analysis shows that the decrease in macrophage density (MD) can be approximated by

\[
MD = C + BA^* 
\]

where C and B are constants, A is the cross-sectional area of the metastasis, and n is the slope of the regression analysis line. For B16-BL6 metastases, \( n = -0.62 ± 0.03 \) (SE) (coefficient of correlation = −0.85, \( P < 0.001 \)), and for 13762NF MTLn3 metastases, \( n = -0.49 ± 0.02 \) (coefficient of correlation = −0.78, \( P < 0.001 \)).

**Macrophage Content of Liver Metastases.** The distribution of macrophages in spontaneous hepatic metastases arising from M5076 reticulum cell sarcoma cells implanted in the inguinal fat pad is shown in Figs. 5 and 6. In common with the above observation on lung metastases, the number of macrophages is...
higher in larger metastases, but the number per unit area is lower. Detailed morphometric measurements on the content of tumor-associated macrophages in individual hepatic metastases arising from the M5076 reticulum cell sarcoma and SJL/J reticulum cell sarcoma are shown in Figs. 7 and 8. The data indicate that the number of macrophages per unit of surface area within liver metastases decreases dramatically and stabilizes at uniformly low levels comparable to those found in the above analysis of lung metastases. Again, the density of intratumoral macrophages can be expressed by the function

$$MD = C + A^n$$

where \( n = -0.36 \pm 0.03 \) (SE) (coefficient of correlation = \(-0.64, P < 0.001\)) for M5076 metastases and where \( n = -0.29 \pm 0.03 \) (coefficient of correlation = \(-0.70, P < 0.001\)) for SJL/J reticulum cell sarcoma metastases.

**DISCUSSION**

The present results indicate that the progressive growth and enlargement of lung and liver metastases, arising from the spontaneous metastasis of a panel of rodent neoplasms of diverse histological lineages, are accompanied by a rapid fall in the density of macrophages within individual metastases. These observations are consistent with our earlier observations on the macrophage content of experimental metastases produced by i.v. inoculation of tumor cells (15). Collectively, these data show that the progressive fall in the density of macrophages within metastases is not limited to specific organs, particular species, or the route of tumor cell dissemination.

Accurate identification of the macrophage content of very small metastatic lesions can be achieved as a result of the novel labeling technique in which iron dextran is used to label tumor-associated macrophages. This method has now been applied successfully to analyze macrophage populations in tumors implanted i.m. and s.c. and to lung and liver metastases arising from a variety of rodent neoplasms: B16 melanoma; Lewis lung carcinoma; M5076 reticulum cell sarcoma; SJL/J reticulum cell sarcomas; and 13762NF mammary adenocarcinoma (15, 16). Although this method cannot detect all tumor-associated...
DENSITY OF MACROPHAGES IN METASTASES

Fig. 6. Light micrograph of a portion of a larger hepatic metastasis from the same liver shown in Fig. 5. The density of macrophages in the field is the lowest illustrated: 208 macrophages/mm². Final magnification, × 200.

Fig. 7. A similar scatter diagram as Fig. 3, in this case for spontaneous hepatic metastases from the M5076 mouse reticulum cell sarcoma. Data for 145 individual metastases are shown. A strong negative correlation between macrophage density and sectional area is seen.

macrophages (for example, nonphagocytic macrophage subpopulations will not be labeled) the technique can identify macrophages throughout large tumor masses (>1-cm diameter). In addition, the relative number of macrophages detected using this method correlates well with data on the relative macrophage contents of tumors determined by other methods (14). Furthermore, because the labeling method exploits phagocytosis, a basic physiological function of macrophages, it has advantages over other labeling methods such as enzyme markers, surface receptors, or antigens which may not be specific to macrophages and are often expressed nonuniformly in macrophage subpopulations and/or require special processing methods (e.g., the use of unfixed frozen sections) for optimal results (16, 20, 21). Similarly, although methods for the direct recovery of macrophages from tumors have been described (13, 14), it is unclear what fraction of macrophages are recovered and how far differences in macrophage age, functional status, and subpopulation heterogeneity affect susceptibility to tissue dispersal methods and influence recovery of viable cells.

Previous investigations have concluded that the macrophage content of tumors is relatively constant with time (13). However, the majority of these studies were conducted on large subcutaneous tumors (>5-mm diameter), and tumor-associated macrophages were quantified following tissue disaggregation. This can result in variable loss of tumor-associated macrophages, and it may also result in contamination by macrophages from adjacent host tissue(s). Furthermore, such methods cannot yet be effectively used in quantifying host and tumor cell populations from very small lesions of the kind examined in this study. The largest metastases evaluated in the current study were <1 mm in diameter, and the macrophage densities in such lesions were already at low levels. Indeed, by the time the metastases have reached cross-sectional areas of 0.2 mm² (corresponding to a diameter of approximately 0.5 mm), the intratumoral macrophage content has already reached this low level. The present observations confirm and extend our earlier findings that the density of macrophages in large tumors does not necessarily correlate with the density of macrophages in micrometastases. These data reinforce the need for caution in concluding that the macrophage content found in large established tumor implants growing i.m. or s.c., albeit more convenient to study, necessarily reflects the true nature of the host response during the earlier stages of tumor development.

The mechanisms responsible for the decrease in the macro-
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phage density in larger metastases are unknown. Previous studies have shown that most tumor-associated macrophages are derived from circulating blood monocytes and that the majority of these cells reside at the host-tumor interface (21, 22). Tumor geometry may be a significant contributing factor to the decrease, particularly if the stimulus for recruitment of macrophages into tumors is related to the surface area of the host-tumor interface, rather than to tumor volume. Tumor geometry, however, does not explain this phenomenon completely. Experiments using B16 melanoma have shown that the removal of a large primary tumor slows the rate of decrease in macrophage density in its metastases (23). This suggests that the presence of a primary tumor may suppress the recruitment of macrophages into distant metastases. Impaired recruitment of macrophages into tumors or other sites of inflammation has been shown by several investigators in tumor-bearing animals (24, 25), and soluble factors released by tumor cells that impair macrophage migration have been identified (26). Alternatively, the primary tumor may serve simply as a “sink” for circulating monocytes. Experiments to measure the rates of recruitment of macrophages into metastases at different stages of growth and the effect of the primary tumor on the recruitment kinetics are in progress.

Finally, the rapid decline in the macrophage content of metastases during their progressive growth and enlargement documented in this study, and in our earlier observations on experimental metastases (15), has potentially important implications for the likely therapeutic utility of biological response modifier agents designed to augment macrophage-mediated responses to established tumors. If the data presented here are representative of events in the progress of human micrometastases, therapy with biological response modifying agents with macrophage activation properties may be of little benefit in eradicating even relatively early metastatic lesions. Even if such agents were to achieve the unlikely goal of activation of all tumor-associated macrophages, and the activated macrophages, in turn, could exhibit maximum killing kinetics, the low ratio of macrophages to tumor cells in lesions containing more than 1000 tumor cells would mean that a significant tumor cell burden would still escape destruction. This phenomenon may well explain previous studies from this and other laboratories (1, 27–29) in which therapy with macrophage activation agents was successful in eradicating established micrometastatic disease in experimental animals but was unsuccessful in treating larger established metastases. These results suggest that agents with macrophage activation activity may be useful in neoadjuvant or adjuvant therapy protocols directed against micrometastases but will not be effective in treating metastatic disease unless used in combination with other therapeutic modalities to reduce the overall tumor burden.

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