Changes in the Macrophage Content of Lung Metastases at Different Stages in Tumor Growth

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The macrophage content of experimental B16 melanoma metastases at different stages of their growth has been quantified with the use of morphometry in conjunction with a recently developed histochemical method for selectively staining intratumoral macrophages. Data are presented from analyses of 954 sections of 155 individual lung metastases, showing that the macrophage content of individual B16 melanoma lung metastases not only varies significantly but also falls dramatically once metastases contain more than 700 tumor cells. In addition to providing new information on host response reactions of micrometastases, these experiments also indicate that conclusions on intratumoral macrophages derived from studies of large primary tumors and metastases in advanced stages of growth may have little or no relevance to events in micrometastases. (Am J Pathol 1985, 118:419–424)

THE WELL-DOCUMENTED ability of activated macrophages to destroy tumor cells *in vitro* has stimulated interest in the possible usefulness of macrophage activation agents in cancer therapy.[1,2] A wide variety of naturally occurring and synthetic molecules have been reported to augment macrophage-mediated destruction of animal tumors, including metastases.[1,3–5] To date, however, this approach has been effective only against minimal tumor burdens. The factors responsible for the lack of success of this approach in enhancing macrophage-mediated destruction of larger tumors are unknown. Unlike chemotherapy and immunologically specific immunotherapy, in which the most common cause of treatment failure is the emergence of resistant tumor cell subpopulations,[6] tumor-cell resistance to macrophage-mediated cytolysis is uncommon.[1,2,5,6] Tumor cells recovered from progressively growing lung metastases in animals treated with macrophage-activating agents remain susceptible to destruction by activated macrophages, at least in vitro.[1,5]

One possible explanation for the failure of nonspecific immunotherapy against all but the most minimal disease is the failure of the host response to keep pace with tumor growth. Information published to date on intratumoral macrophage populations has come exclusively from studies on large primary tumors and macroscopic metastases.[7,8] Although the macrophage content of large tumors is relatively constant, nothing is known about macrophage reactions in "early" micrometastases. Recently we developed a new histochemical method that allows reliable identification of macrophages in paraffin sections of micrometastases.[9] In this article we report the use of this technique in conjunction with morphometry in examining the number and distribution of macrophages in metastases at different stages in their growth.

Materials and Methods

Animals and Experimental Metastases

Eight- to 12-week-old female C57BL/6J mice were obtained from the Laboratory Animal Sciences Division of Smith Kline and French Laboratories and were provided with pelleted food and tap water *ad libitum*.

B16-F1 murine melanoma cells were maintained in vitro in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and D-glucose (1000 mg/l) and sodium pyruvate, supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY) as described previously.[9]

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Single-cell suspensions were obtained by treating monolayers with 0.25% trypsin and 0.01% EDTA for 1 minute (GIBCO).

Experimental lung metastases were produced by injection of 1×10^5 viable B16-F1 melanoma cells into the tail vein as described previously.[9] The B16 melanoma, a syngeneic tumor of spontaneous origin, was selected for study for two reasons. First, it has been selected for routine use by the National Cancer Institute in screening for new biologic response modifier (BRM) agents designed to enhance macrophage function, and the ability to accurately quantify effects on intratumoral macrophage content will be an important criterion in a comparison of the potency of different BRM agents.[10] Second, the macrophage content of this tumor is low, and it thus offers a more demanding challenge in evaluating the sensitivity of methods for macrophage detection.[7] This situation is also more relevant to human tumors, many of which are characterized by a low macrophage content, at least when compared with the very high macrophage content (30–50% total cells) reported in highly antigenic tumors induced on oncogenic viruses or chemical carcinogens.[2,7,8,11]

Staining Procedures

To selectively label tumor associated macrophages, we gave tumor-bearing mice intravenous (tail vein) injections of colloidal iron–dextran complexes 8, 14, or 22 days after tumor cell injection and sacrificed the mice 24 hours after the injection of colloidal iron, as described previously.[9] We have shown that this procedure labels tumor associated macrophages with high efficiency in subcutaneous and intramuscular tumors and lung metastases.[9] Tumor-bearing lungs were fixed in phosphate-buffered formalin and processed for routine paraffin sections. Melanin pigment and endogenous peroxidase activity were removed by treating the sections with 10% hydrogen peroxide in methanol for 24 hours. To selectively stain macrophages, phagocytosed colloidal iron was first converted to Prussian blue by incubating the sections with 1% potassium ferrocyanide in 1% HCl for 20 minutes at room temperature. The pseudoperoxidase activity of Prussian blue was then used to catalyze the oxidation of diaminobenzidine by hydrogen peroxide.[12] The sections were incubated in a medium containing 50 mg 3,3'-diaminobenzidine HCl (Electron Microscopy Sciences, Ft. Washington, PA) and 10 ml 3% H_2O_2 in 100 ml 0.1 M Tris HCl buffer (pH 7.4), for 20 minutes at room temperature and washed in three changes of distilled H_2O. To enhance the staining reaction, sections were exposed to osmium tetroxide vapor for 15 minutes, then counterstained with 1% methyl green, dehydrated with alcohols, and coverslipped. Macrophages stain brownish black, and tumor cells stain green.

Morphometric Analysis

The density of macrophages within individual metastases was determined by expressing the number of macrophages in individual metastases in relation to the cross-sectional area of that lesion, as follows:

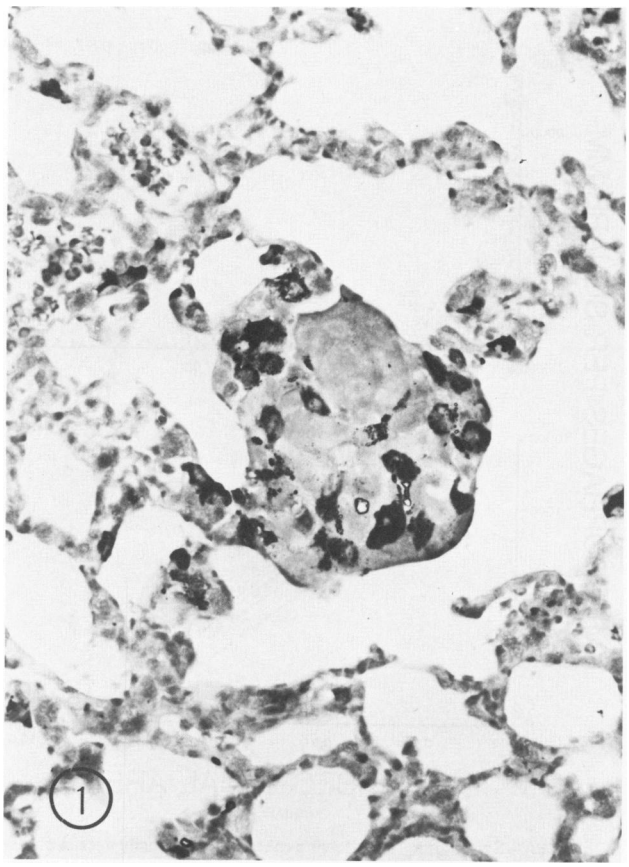
$$\text{Macrophage density (no./sq mm)} = \frac{\text{Number of macrophages in a metastasis section}}{\text{Cross-sectional area of the metastasis (sq mm)}}$$

The cross-sectional area of metastases was measured and the macrophage density was calculated with a semiautomated image analysis unit (Zeiss Videoplan, Carl Zeiss, Inc., New York, NY) directly from a Zeiss microscope equipped with a drawing tube. Sections were prepared from the lungs of 9 mice, and cross-sections from 155 metastases were measured. We examined up to eight serial sections of the same metastasis to limit sampling error in analysis of data from individual lesions. A total of 954 sections of metastases was examined.

The volume density of tumor cell nuclei (VV_n) in micrometastases produced by B16-F1 melanoma cells was determined from area-perimeter analysis of hematoxylin and eosin-stained sections from the same lungs used for the macrophage density determinations. The volume density of tumor cell nuclei was calculated with the use of the commercially available stereology program for the Videoplan (Carl Zeiss, Inc.) The total number of tumor cells in individual metastases was then calculated from the volume density of nuclei, and the measured cross-sectional area (A) was calculated by treating the lesion as an idealized sphere as follows:

$$\text{Number of tumor cells} = VV_n \, 4/3\pi \left[\sqrt{\frac{A}{\pi}}\right]^3$$

Figure 1—Cross-section of mouse lung 9 days after intravenous injection of B16 melanoma cells and the section stained as described in the text to reveal macrophages (black cells) in a micrometastasis. There are 25 staining cells in the lesion, which has an area of 0.008 sq mm. The density of macrophages is 2992 macrophages/sq mm. (×1000) **Figure 2**—Another lung metastasis from the same animal shown in Figure 1. There are 25 macrophages in the lesion. In this case, however, the area of the lesion is 0.035 sq mm, and the density of macrophages is only 709 macrophages/sq mm. Also note that most of the macrophages are confined to the peripheral regions of the lesion. (×1000) **Figure 3**—An experimental lung metastasis from a mouse sacrificed 22 days after injection of tumor cells. Macrophages were specifically stained as described in the text. There are 156 staining cells in the lesion, which has an area of 0.693 sq mm. The density of macrophages is 225 macrophages/sq mm. (×250) **Figure 4**—An experimental lung metastasis from the same lung shown in Figure 1. Marked heterogeneity in the macrophage content of the different regions (*A* and *B*) is evident. In both regions, however, most macrophages lie along the edge of the lesion. (×100)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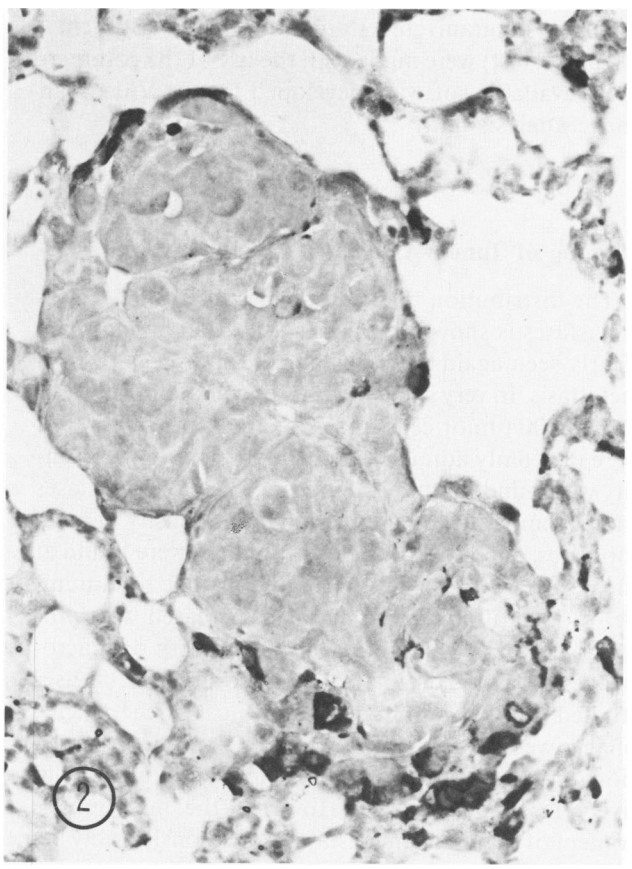
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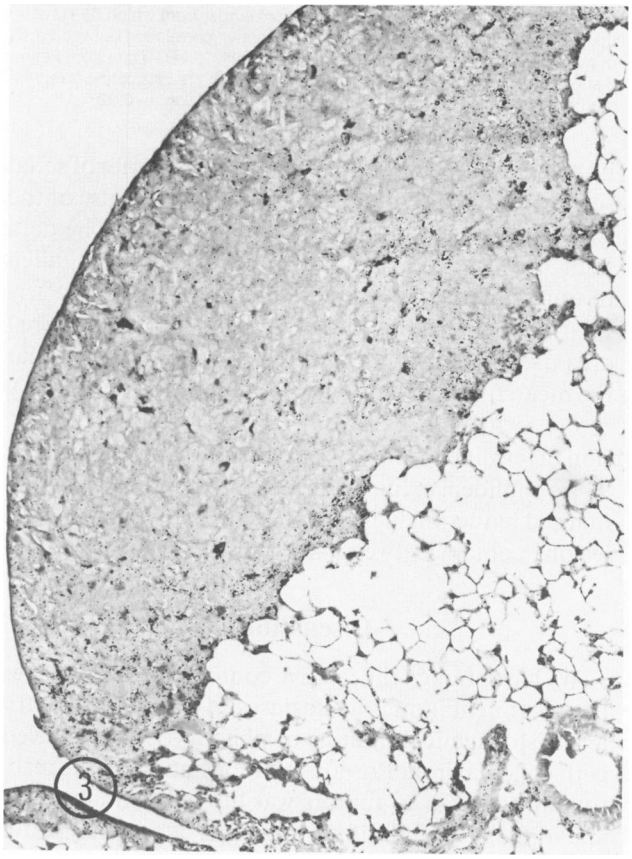
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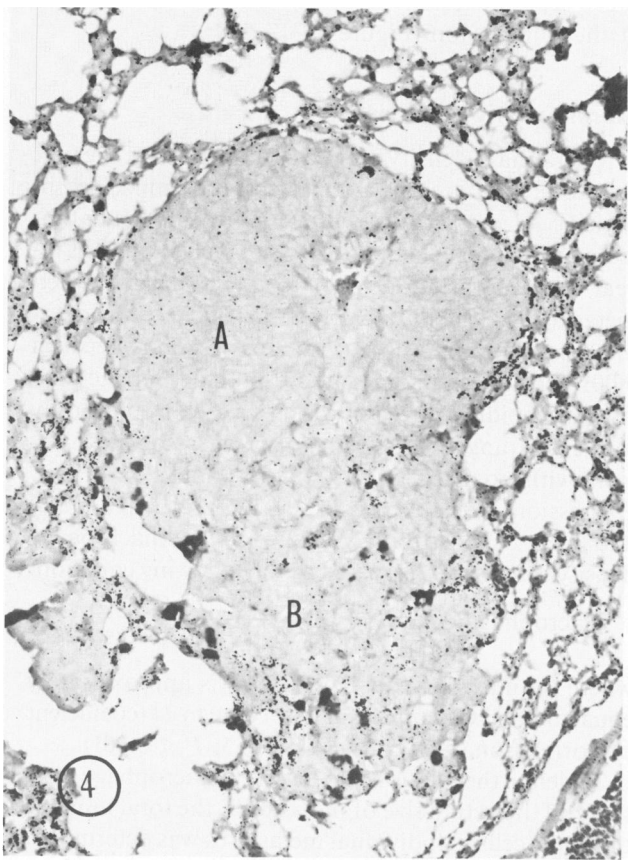
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4

Scatter diagrams and statistical analysis (Student *t* test and *F* test) were made with the use of the commercially available software developed for the Videoplan image analysis unit.

Results

Staining of Tumor-Associated Macrophages

The distribution of macrophages in B16-F1 micrometastases is shown in Figures 1–4. Macrophages are clearly seen against the unstained tumor cells in each metastasis. In very small metastases containing between 10 and 700 tumor cells, macrophages and tumor cells were randomly admixed, and a high macrophage density was found (Figure 1). Further growth of metastases was accompanied by a reduction in the density of macrophages, and most of the macrophages were found at the periphery of the lesion (Figure 2). The total number of intratumoral macrophages increased with progressive growth (Figure 3), but the density of macrophages decreased dramatically. In the larger metastases studied (up to 1 mm in diameter) most of the macrophages were located at the expanding margin of the lesion, and the central portions typically contained relatively few macrophages. However, the macrophage content of individual metastases varies considerably between separate lesions as well as within different parts of the same lesion (Figure 4).

Morphometric Analysis

Macrophage density plotted as a function of the unit cross-sectional area of a metastasis is summarized in Figure 5. Each point in Figure 5 represents the datum from a separate metastasis cross-section. The results reveal marked variation in macrophage density when metastases are small (ranging from 8000 to 100 macrophages/sq mm in lesions with cross-sections between 0.001 and 0.01 sq mm). In addition, macrophage density falls rapidly in larger metastases, reaching uniformly low levels (range, 1000 to 5 macrophages/sq mm) in lesions with cross-sections of between 0.1 and 1.0 sq mm. Expression of the data as a log-log plot (Figure 5B) and linear regression analysis revealed that macrophage density can be approximated by the following equation:

$$\text{Macrophage density} = B + \frac{C}{\text{Area of metastasis}}$$

where B and C are constants and B is approximately equal to 5 and C is approximately equal to 24 (coefficient of correlation, -0.82; $F = 1956.55$; $P < 0.001$).

To define the relationship between macrophage density and the actual size of metastases, the total number of tumor cells in individual metastases was determined

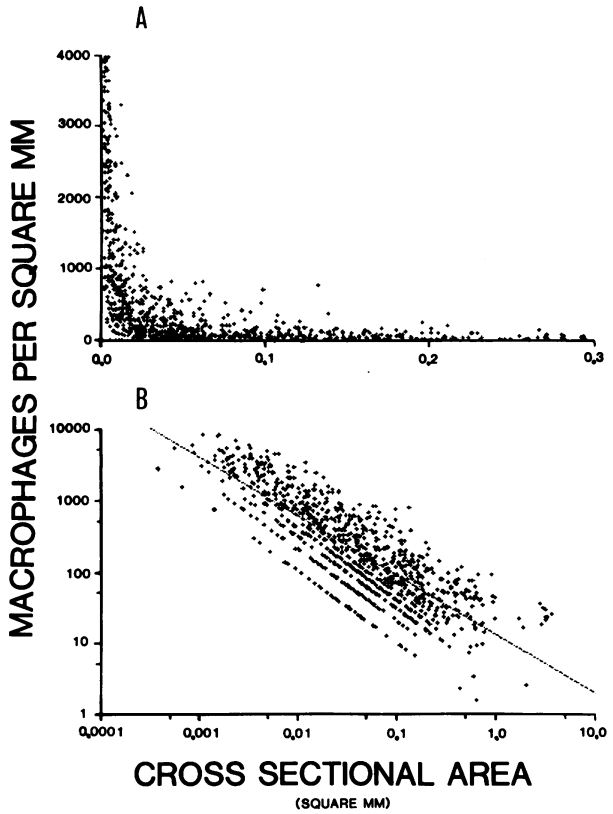

Figure 5A—Scatter diagram showing macrophage density versus cross-sectional area. Each point represents a section from an individual metastasis. Data were collected from nine sets of lungs, from which 954 metastases sections were evaluated. A strong negative correlation between area of metastasis and macrophage density is evident. **B**—Data from Figure 3 shown in a log–log plot. The dotted line shows the results of linear regression analysis of these data. Coefficient of correlation, −0.82.

by area-perimeter stereologic analysis of a series of serial sections from individual metastases with the use of the Zeiss Videoplan automated image analyzer. The data in Figure 5 indicate that macrophage density has fallen to uniformly low levels in *all* metastases examined by the time they have achieved a median cross-sectional area of 0.01 sq mm. Stereologic analysis revealed that the mean tumor cell density in lesions of this size is 0.9 $\pm$ 0.4 $\times$ 10^3 cells/cu μ (mean value $\pm$ standard deviation determined from measurements on 40 metastases; 93% confidence limit). These data indicate that a marked reduction in macrophage content occurs when lesions contain between 400 and 1000 tumor cells.

Discussion

We have studied the relative content of macrophages in experimental micrometastases using morphometry and histochemical staining of phagocytic cells labeled with colloidal iron. Previously, information on the macrophage content of tumors was limited to analyses of macrophage populations recovered after enzymatic

and/or mechanical disaggregation of large macroscopic tumors produced by subcutaneous or intramuscular injection of large numbers of tumor cells.[7,8] Such studies have shown that the macrophage content of tumors varies widely between different tumors, with higher macrophage content being more common in highly antigenic tumors.[8,11] It has been suggested, however, that the macrophage content of a given tumor is characteristic and relatively constant over the growth of the tumor.[8,11] The present results do not support this generalization.

Unfortunately, current cellular isolation methods are suitable only for use with large tumors; and previous efforts to quantify the macrophage content of tumors have had, of necessity, used subcutaneous tumors of substantial size that typically exceeded 1 g or more in weight.[8] Furthermore, tissue disaggregation procedures do not differentiate tumor-associated macrophages from those present in any host tissue excised at the tumor margin. Also, such techniques are hindered by the variable, and often unknown, degrees of cell loss (both tumor cells and macrophages) produced by the tissue disaggregation technique(s). Similarly, histologic studies of the macrophage content of tumors have been limited exclusively to examinations of large primary tumors and advanced metastases.[7,8,11,13-15] Our results showing that the macrophage content of small metastases is highly variable and declines rapidly to uniformly low levels in larger tumors indicate that observations made on large tumors cannot be reliably extrapolated to events in early growth of metastases. We have found a similar correlation in micrometastases from a variety of rodent tumors (B16, B16 BL-6, Lewis lung, M5076, SJL reticulum-cell sarcomas in mice and 13762 mammary adrenocarcinomas in rats; data not shown).

The present method, in common with all others used to identify intratumoral macrophages, may fail to detect particular subsets of macrophages that do not express the properties being assayed. However, other phenotypic markers for macrophages such as enzymes or antigens are known to vary with changes in macrophage maturation and/or activation.[16,17] In contrast, phagocytosis of particulate materials, which forms the basis of the current assay, is a *basal* function of macrophages, which continues to be expressed when other properties, eg, oxidative burst and microbicidal and tumoricidal activities, are not expressed.[2,16] It is also acknowledged that the method used here requires uniform delivery of the colloid throughout the tumor. Once again, this deficiency applies to all efforts to identify intratumoral host cells using systemically administered labels. However, the extremely small particle size of iron dextran (~6 nm) should favor a large volume of tissue distribution, especially in tumors where vascular permeability is high and intracellular junctions are poorly formed. Equally important, however, is the fact that failure to label all macrophages with this technique would also mean that delivery of systemically administered therapeutic agents to macrophages to stimulate their tumoricidal activity would also have a low probability of success.

The failure of immunotherapy to eradicate all but minimal tumor burdens is well documented.[1] The present work demonstrates an inverse correlation between size of metastases with macrophage content in the B16 melanoma and suggests that the failure of nonspecific immunotherapy of this tumor may be related to the low macrophage density in metastases larger than 1000 cells. Assuming exponential cell growth within metastases and a cell doubling time of 21 hours *in vivo*,[18] it would take approximately 1 week for a metastasis arising from a single cell to reach this size. Interestingly, this is precisely the time reported for the onset of failure of immunotherapy of metastases of the B16 melanoma.[1,2] It is not known whether a similar correlation exists for human tumors. Although a strong host response has been associated with a better prognosis for certain human tumors,[13,19] the mere presence of large numbers of macrophages in a tumor is not necessarily indicative of a meaningful host response.[1] The rate of recruitment of new macrophages into the tumor and the tumoricidal capacity of the recruited macrophages may be more important than total macrophage content. It has been shown, for example, that macrophages are susceptible to lymphokine-mediated activation to the tumoricidal state for only a few days after emigrating from the bloodstream.[17] The same study also showed that tumoricidal activity also decays rapidly within 2 or 3 days and that subpopulations of previously tumoricidal macrophages may be refractory to a second cycle of activation by lymphokines released by T-lymphocytes.[17] This implies that for immunotherapy to be successful, the rate of recruitment of new macrophages that can be activated to the tumoricidal state must be high if an effective tumoricidal response is to be mounted. The present results suggest that for the B16 melanoma either macrophage recruitment fails to keep pace with tumor expansion or the rate of macrophage recruitment falls when metastases attain a certain size. Although the present experiments do not distinguish between these possibilities, several studies have reported reduced recruitment of macrophages into tissues in tumor-bearing animals and that certain tumor cells, including the B16 melanoma, secrete materials that impede macrophage migration from circulation.[20,21] Studies designed to address the rate of recruitment of macrophages into lung metastases using the techniques described here are now in progress.

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