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Macrophage Content of Murine Sarcomas and Carcinomas: Associations with Tumor Growth Parameters and Tumor Radiocurability[1]

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ABSTRACT

Experiments were designed to investigate whether the tumor-associated macrophage (TAM) content of murine solid tumors correlates with the clonogenic ability of tumor cells to establish s.c. tumors, tumor growth rate, extent of tumor necrosis, tumor metastatic propensity, and tumor radioresponse. Of 13 tumors studied, 6 were sarcomas and 7 were carcinomas; all tumors were of spontaneous origin in C3Hf/Kam mice, with the exception of one sarcoma that was induced by 3-methylcholanthrene. Tumors were growing in the hind thighs of syngeneic mice, and their TAM content was determined when they were 8 mm in diameter. The TAM content varied greatly among tumors, ranging from 9 to 83%. Tumor bearing mice experienced a reduction of 50% or more in the number of peritoneal macrophages, but the degree of reduction was independent of TAM content. A significant negative correlation was noted between TAM content and TD_{50} values (*i.e.*, the number of tumor cells needed to produce tumors in 50% of injected sites) and between TAM content and the amount of tumor necrosis. Also, an obvious trend toward positive correlation between TAM content and reduced local tumor radiocurability was apparent. No correlation was found between TAM content and tumor growth rate or metastatic spread. TAM from the NFSA sarcoma (a tumor with a low TD_{50} value, almost without necrosis, and poorly responsive to radiation) stimulated the *in vitro* growth of NFSA tumor cells. These observations suggest that high TAM content could be conducive to tumor cell proliferation and could be a factor in poor tumor radioresponse.

INTRODUCTION

Participation in host defense against tumors is one of the many functions of macrophages (1, 2). Macrophages are involved in both the afferent and efferent arms of antitumor immune response. When activated by any of a number of stimuli, macrophages become capable of recognizing cells of various tumor types, and they acquire the ability to be cytostatic or cytotoxic to neoplastic cells (2, 3). Studies on the interaction of macrophages with tumor cells, performed both *in vitro* and *in vivo*, have commonly used activated macrophages and have almost invariably demonstrated that macrophages exhibit tumor cell-destroying properties (2–4). Such studies are the basis for the currently prevalent thinking that macrophages play an important role in restricting tumor development and progression. This implies that tumor infiltration by macrophages should be beneficial to the tumor host. Earlier studies of TAM,[4] which used rat sarcomas, supported this contention by providing evidence that tumors with a high macrophage content were less metastatic than tumors with a low macrophage content (5, 6). Tumors with a high macrophage content were also more immunogenic. A number of subsequent studies have provided clear evidence that TAM react with tumor cells. For example, whereas macrophages recovered from an immunogenic nonmetastasizing murine sarcoma were cytotoxic, those from a weakly immunogenic metastasizing variant of the same tumor were stimulatory to tumor cell growth (7). Similarly, macrophages derived from regressing Maloney sarcomas were found to be cytotoxic, whereas those from progressing sarcomas exerted no influence on tumor cell growth (8). Thus, tumor-residing macrophages can destroy tumor cells, stimulate tumor cell proliferation, or be ineffective. However, more recent studies on the role of TAM showed no correlation between the macrophage content and the metastatic propensity of several murine and rat tumors, including tumor variants obtained from metastases of these tumors (9). Therefore, despite extensive investigation, the role of macrophage infiltration in the metastatic behavior of malignant tumors remains uncertain.

Interestingly, little information is available on the association between the extent of macrophage infiltration and tumor progression parameters other than metastatic spread, and between TAM content and tumor response to therapy. The experiments reported here were designed to determine whether the macrophage content of 13 different murine tumors, 6 sarcomas and 7 carcinomas, correlated with tumor take, tumor growth rate, extent of tumor necrosis, and metastatic propensity as well as with tumor radioresponse.

MATERIALS AND METHODS

Mice. Inbred C3Hf/Kam mice, bred and maintained in our specific pathogen-free colony, were used. They were 9–17 weeks old at the start of experiments, with the exception of the group of animals used to determine macrophage content in the peritoneal cavity, which were about 24 weeks old. Within each experiment, mice were of the same sex and were housed 5–7/cage.

Tumors. Thirteen different tumors, of which 6 were sarcomas and 7 were carcinomas, syngeneic to C3Hf/Kam mice were used. The sarcomas consisted of 3 fibrosarcomas, designated FSA, FSA-II, and NFSA and 3 unclassified sarcomas, designated SA-IIA, SA-NH, and SA-4020. The carcinomas included 4 mammary carcinomas, designated MCA-4, MCA-29, MCA-35, and MCA-K; a hepatic carcinoma, designated HCA-I; an ovarian carcinoma, designated OCA-I; and an adenocarcinoma of the salivary gland, designated ACA-SG. All tumors were spontaneous in origin, with the exception of FSA, which was induced by 3-methylcholanthrene (10). These tumors have been described in detail elsewhere (11).

Tumors were generated either i.m. in the right hind thighs or s.c. over the abdomen, depending on the purpose of the experiment. The number of tumor cells injected to generate tumors varied greatly and will be listed at appropriate places later in the text. Single-cell tumor suspensions were obtained by enzymatic digestion of tumor minces using 0.025% trypsin and DNase (12).

Determination of Tumor Macrophages. Tumors used for TAM analysis were 8 mm in diameter. They were generated by injecting 4.4×10^5–7×10^5 viable tumor cells into the muscles of the right hind thighs. The assay used to determine TAM content has been described in detail by Moore and McBride (13). Macrophages in tumor cell suspensions

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[4] The abbreviations used are: TAM, tumor-associated macrophages; TD_{50}, number of tumor cells required to produce s.c. tumors in 50% of injected sites; TCD_{50}, dose of radiation yielding local tumor control in 50% of animals; TDDT, tumor diameter doubling time; PE, peritoneal exudate.

were identified by their ability to form rosettes after incubation with sensitized sheep erythrocytes. The erythrocytes were sensitized with rabbit anti-sheep RBC antibody; 0.1 ml of tumor cells at 10^6/ml was added to 0.1 ml of 1.7% sensitized RBC, spun, and incubated for 30 min at room temperature. The percentage of macrophages in the suspension was determined after staining nucleated cells with crystal violet. The same method was used to identify macrophages in the peritoneal cavity of normal mice and in mice bearing 8-mm tumors in the leg. Enzymatic methods used for tumor disaggregation, including the one which we used here, have been found to have little if any influence on the cellular composition of tumors (13–16). Thus, the proportion of macrophages in disaggregated tumor cell suspensions is considered to be a true representation of cellular composition of undigested tumors.

s.c. Tumor Take. This assay has been described in detail (17). Mice were given s.c. injections at 4 sites overlying the abdomen with various numbers of viable tumor cells diluted in 0.05 ml of Hsu's medium. Thereafter, mice were examined 2–3 times a week for tumor development. Tumor take was expressed as the TD_{50} value.

TDDT. Tumors were generated by injecting 5×10^5 viable cells into the hind thighs of mice. Mice were inspected for tumor appearance and growth 3 times a week. Tumor growth was determined by measuring 3 mutually orthogonal tumor diameters with a vernier caliper. The number of days tumors needed to grow from 6–12 mm in diameter was used as the TDDT.

Determination of Tumor Necrosis. Tumors 8 mm in diameter growing in the right thighs of mice were excised, fixed in 10% phosphate-buffered formalin solution, embedded in paraffin wax for histological sectioning, and then stained with hematoxylin and eosin. Necrosis was assessed by light microscopy using an adaptation of the Chalkley Point method (18).

Spontaneous Metastasis. Solitary tumors in the legs were generated by injecting 5×10^5 or 10^6 viable tumor cells in a volume of 10–20 μl into the right thighs of mice. The tumor-bearing legs were removed surgically when tumors grew to 12 mm in diameter. Spontaneous metastases were scored at 14–120 days after tumor removal, depending on the tumor type. As indicated elsewhere (11) the point at which the number of spontaneous metastases reached a plateau was considered to be the optimal time to determine metastases. To do so, mice were killed and their lungs removed and fixed in Bouin's solution. Metastases were seen as white round nodules on the surface of the yellowish lung and were counted with the naked eye or when needed with a magnifying glass.

Tumor Response to Irradiation. The TCD_{50} assay was used to assess tumor response to ionizing radiation. The mice were given injections in the right hind thighs of 5×10^5 tumor cells. When tumors had grown to 8 mm in diameter, the tumor-bearing leg was exposed to a single dose of γ-radiation, which was delivered from a dual-source ^{137}Cs unit at a dose rate of 825 rads/min. The mice were not anesthetized during irradiation but were immobilized in a jig. The tumor was centered in the circular radiation field, 3 cm in diameter. Mice were checked for the presence of tumor at the irradiated site at 9- to 12-day intervals for up to 120 days. TCD_{50} values were computed by the logit method of analysis (19).

***In Vitro* Effect of TAM or Peritoneal Macrophages on Tumor Growth.** Macrophages were separated from NFSA tumors from 8–12 mm using Renografin gradients at a density of 1.11 (20). The separated cell population contained 95% macrophages. PE cells were derived from mice as described previously (21). Four $\times$ 10^6 TAM or PE cells suspended in Hsu's medium were plated into 35-mm Petri dishes and incubated for 2 h, at which time unattached cells were removed by vigorous rinsing with fresh medium. More than 60% of PE cells remained attached, and by morphological criteria they were determined to be macrophages. Less than 5% of the cells in TAM suspension did not attach. NFSA cells (10^5) which were from the fifth *in vitro* passage and contained no macrophages were added onto macrophage monolayers and incubated for 2 days. At this time [^{3}H]thymidine was added to the dishes at a concentration of 0.1 μCi/ml of medium, and the cells were incubated for an additional 1 h. The medium was then replaced with 5 ml of phosphate-buffered saline and the cells harvested by scraping. The cell suspension was transferred to a fiberglass filter (GF/C) on a vacuum aparatus. The cells were washed with 10 ml of cold PBS followed by 10 ml of cold 5% perchloric acid and then 5 ml of cold ethanol. Each filter was then transferred to a scintillation vial, digested with a tissue solubilizer (NCS; Amersham–Searle), and counted for tritium content in a liquid scintillation counter.

Statistical Evaluation. Correlation coefficients for tumor macrophage content and other variables were calculated by simple linear correlation and tested for significance at the 5% level.

RESULTS

TAM Content in Sarcomas and Carcinomas. Table 1 shows the percentage of TAM in murine sarcomas and carcinomas growing as solitary i.m. tumors in the thigh. Tumors were 8 mm in diameter when assessed for macrophage content. The percentage of TAM varied greatly among tumors. The range was 26–83% for sarcomas and 9–74% for carcinomas. The average TAM content was 44% in sarcomas and 39% in carcinomas.

We further investigated whether the growth of solitary tumors influenced the macrophage content in the peritoneal cavity. Mice bearing 8-mm diameter tumors with the highest percentage of macrophages (NFSA and HCA-I) or 8-mm diameter tumors with a much lower percentage of macrophages (FSA and MCA-4) were used. The results are presented in Table 2. Untreated mice had 4.4 ± 0.5 (SE) $\times$ 10^6 PE cells, of which 63% were macrophages. The total number of PE cells was not altered by the presence of the 2 sarcomas but was slightly decreased in mice bearing the 2 carcinomas. However, the percentage of PE macrophages was significantly lower in mice bearing any of the 4 tumors than in normal mice. In each instance the total number of PE macrophages was 50% or less than the control value, implying that the decrease did not depend on the extent of tumor infiltration with macrophages.

Correlation between TAM Content and Tumor Take, Tumor Growth, and Extent of Tumor Necrosis. A number of growth

Table 1 *Percentage of macrophages in C3Hf/Kam mouse sarcomas and carcinomas of 8 mm diameter*

Tumors were located in the right hind thighs of mice. Macrophages were identified by the Fc receptor technique. Six tumors of each tumor type were analyzed for TAM. Tumors of each type were generated from a single suspension; all 6 tumors were analyzed on a single day in the case of FSA, FSA-II, MCA-29, and MCA-35 tumors, but for the remaining tumors, analysis of TAM was performed within a period of 2–11 days.

	% of TAM		
	Sarcomas		Carcinomas
FSA	27.0 ± 1.8[a]	MCA-4	31.2 ± 2.0
FSA-II	26.9 ± 3.2	MCA-29	34.1 ± 1.9
NFSA	82.8 ± 3.4	MCA-35	52.7 ± 4.6
SA-NH	58.9 ± 2.4	MCA-K	37.8 ± 3.7
SA-4020	25.8 ± 1.1	HCA-I	74.1 ± 2.5
SA-IIA	42.0 ± 6.5	OCA-I	8.7 ± 1.4
		ACA-SG	33.9 ± 2.3
Total	43.9 ± 9.34		38.9 ± 7.61

[a] Mean ± SE.

Table 2 *Effect of 8-mm diameter solitary tumors on the number of PE cells and percentage of PE cells that were macrophages*

Tumors were located in the right hind thighs of mice. Macrophages were identified by the Fc receptor technique. Groups consisted of 4 mice each.

Mice and tumor	No. of PE cells ($\times 10^6$)	% of macrophages in PE cells
Normal mice	4.4 ± 0.5[a]	62.9 ± 3.7
Mice bearing NFSA	4.8 ± 0.5	20.5 ± 2.5
Mice bearing HCA-I	3.0 ± 0.8	48.6 ± 3.4
Mice bearing FSA	4.1 ± 0.4	22.7 ± 3.0
Mice bearing MCA-4	3.7 ± 0.3	26.7 ± 4.2

[a] Mean ± SE.

characteristics of the 6 sarcomas and 7 carcinomas were assessed and were correlated with the TAM content of the tumors. The ability of tumor cells to generate s.c. tumors, estimated by the TD_{50}, varied greatly among tumors, with a range in TD_{50} values from only 29 cells for NFSA to 9.2×10^4 cells for OCA-I. In general, sarcomas had lower TD_{50} values than did carcinomas. Individual TD_{50} values for all 13 tumors have already been reported (11). To determine whether the s.c. take ability of cells derived from these tumors correlated with the extent of TAM infiltration, we plotted TD_{50} values *versus* percentage of TAM in tumors and calculated correlation coefficients (Fig. 1). A statistically significant negative correlation of −0.56 was obtained ($P < 0.05$), indicating that tumors with lower TD_{50} values had a higher macrophage content. This finding suggests that the presence of macrophages in tumor cell suspension is conducive to the establishment of tumor growth. The correlation coefficients for sarcomas and carcinomas separately were −0.37 and −0.72, respectively. These correlations were not statistically significant.

Tumor growth rate was determined by the TDDT. Results presented in Table 3 show that doubling times were relatively similar for most sarcomas, ranging from 5.3–7.2 days, with the exception of SA-NH, which grew somewhat more slowly (9.8 days). In contrast, carcinomas showed greater variability in doubling times than did sarcomas. Whereas the doubling times of 4 carcinomas were between 7.1 and 8.9 days, the doubling times of the remaining 3 carcinomas were between 18.9 and 23.7 days. Fig. 2 shows the relationship between tumor doubling times and TAM content. No significant correlations were found between TAM infiltration and the doubling times of sarcomas or carcinomas, or even both, the correlation coefficients being 0.22, −0.19, and −0.15, respectively.

Tumors of 8 mm diameter were analyzed for the extent of necrosis (Table 3). In general, sarcomas of this size contained only small areas of necrosis. The percentage of necrotic tissue ranged from 0.2% for NFSA to 3.6% for SA-NH. However, 8-mm diameter carcinomas showed a greater variation in the extent of necrosis, ranging from 0.5% for HCA-I to 21.6% for OCA-I tumors. Fig. 3 shows a negative correlation between the percentage of necrosis and the macrophage content, indicating that tumors with a higher macrophage content were less necrotic. Correlation coefficients were statistically significant

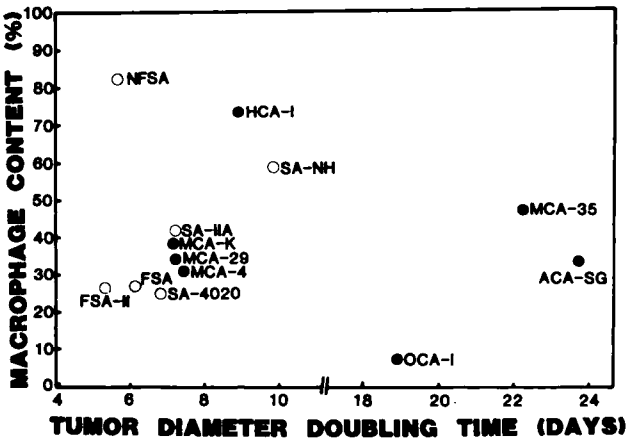

Fig. 2. Correlation between tumor diameter doubling time of murine sarcomas (○) or carcinomas (●) and TAM content. Tumor diameter doubling time is taken as the time in days tumors required to grow from 6–12 mm in diameter. Correlation coefficients are 0.22 for sarcomas, −0.19 for carcinomas, and −0.15 for all tumors tested.

Table 3 *Growth rate, percentage of necrosis, and radioresponse of sarcomas and carcinomas in C3Hf/Kam mice*

	Tumor diameter doubling time (days)[a]	% necrosis in 8-mm diameter tumors[b]	TCD_{50} (Gy) of 8-mm diameter tumors (95% confidence limits range in parentheses)[c]
Sarcomas			
FSA	6.1 ± 0.2[d]	1.9 ± 1.3	42.1 (38.0–46.6)
FSA-II	5.3 ± 0.3	2.6 ± 0.9	74.8 (68.6–81.6)
NFSA	5.7 ± 0.2	0.2 ± 0.2	80.5 (77.6–83.6)
SA-NH	9.8 ± 0.5	3.6 ± 1.5	49.3 (44.9–54.0)
SA-4020	6.7 ± 0.5	3.3 ± 2.4	77.0 (71.2–83.4)
SA-IIA	7.2 ± 0.6	0.6 ± 0.3	61.6 (56.0–67.8)
Carcinomas			
MCA-4	7.4 ± 0.5	4.7 ± 1.7	58.6 (52.0–65.6)
MCA-29	7.2 ± 0.3	2.5 ± 0.8	62.3 (56.7–68.5)
MCA-35	22.2 ± 3.5	4.3 ± 2.3	58.9 (53.3–65.1)
MCA-K	7.1 ± 0.4	9.5 ± 3.4	48.2 (45.0–50.6)
HCA-I	8.9 ± 0.5	0.5 ± 0.2	>81.0
OCA-I	18.9 ± 1.3	21.6 ± 0.6	52.6 (49.3–56.0)
ACA-SG	23.7 ± 3.1	6.7 ± 4.0	65.6 (57.7–74.1)

[a] Tumor diameter doubling time was expressed as the time needed for tumors to grow from 6–12 mm in diameter. Groups consisted of 9–20 mice each.
[b] Groups consisted of 4 tumors each.
[c] Between 40 and 50 mice were used in each TCD_{50} assay.
[d] Mean ± SE.

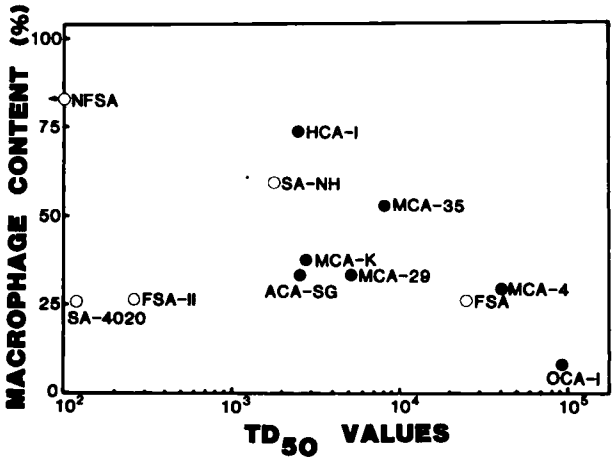

Fig. 1. Correlation between TD_{50} values for murine sarcomas (○) or carcinomas (●) and TAM content. Individual TD_{50} values ± SE have been reported in Ref. 11. Correlation coefficients are −0.37 for sarcomas, −0.72 for carcinomas, and −0.56 for all tumors tested. The latter value is statistically significant at $P < 0.05$.

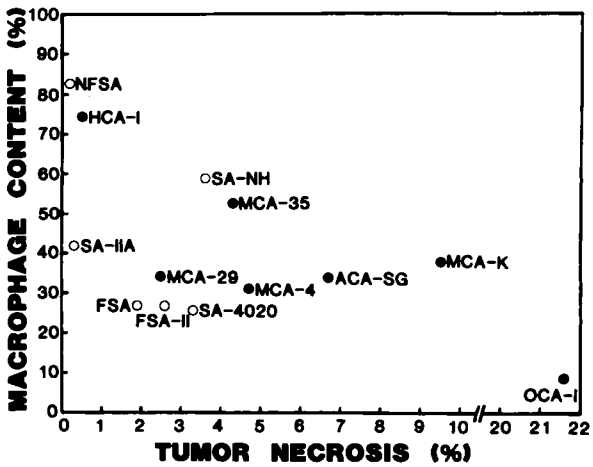

Fig. 3. Correlation between the extent of necrosis in murine sarcomas (○) or carcinomas (●) and TAM content. Correlation coefficents are −0.47 for sarcomas, −0.79 ($P < 0.05$) for carcinomas, and −0.58 ($P < 0.05$) for all tumors tested.

when all tumors were used in the assessment ($r = -0.58$; $P < 0.05$), or only carcinomas $r = -0.79$; $P < 0.05$), but were not significant when only sarcomas were assessed ($r = 0.47$).

Correlation between TAM Content and Spontaneous Metastases. The relationship between the incidence of spontaneous metastasis and macrophage content is plotted in Fig. 4. The metastasis incidence ranged from 0% (SA-IIA and MCA-4) to 100% (SA-NH, HCA-I, and OCA-I). No correlation was found between the metastasis incidence and macrophage content; correlation coefficients were −0.16 for sarcomas, 0.14 for carcinomas, and −0.02 for all tumors tested. Inserted in parentheses in Fig. 4 are the mean numbers of metastases for each tumor type. Interestingly, with 5 highly metastatic tumors (OCA-I, SA-4020, MCA-K, SA-NH, and HCA-I), the number of metastases per lung increased as the number of macrophages in the primary tumor increased. The incidence, mean number, and range of lung metastases for each individual tumor type were reported earlier (11).

Correlation between TAM Content and Tumor Radioresponse. The radioresponse of 8-mm diameter sarcomas and carcinomas, assessed by the TCD_{50}, is shown in Table 3. TCD_{50} values for single doses of γ-rays ranged from 42.1 Gy (FSA) to more than 80 Gy (HCA-I). The ranges in radioresponse were similar for sarcomas and carcinomas. The relationship between tumor radioresponse and the extent of TAM infiltrates is shown in Fig. 5. Correlation coefficients were 0.21 for sarcomas, 0.73 for carcinomas, and 0.43 for all tumors tested. Although these values are not statistically significant, the clear tendency is for tumors with a higher TAM content, especially carcinomas, to be less radiocurable.

***In Vitro* Interaction of TAM from NFSA Tumors with Tumor Cells.** To test whether TAM interact with corresponding tumor cells, we assessed TAM activity from the most radioresistant sarcoma, NFSA. Results presented in Table 4 show that TAM markedly stimulated the *in vitro* growth of NFSA tumor cells. Peritoneal macrophages from normal mice were also stimulatory but to a lesser degree than TAM.

DISCUSSION

Our results show that TAM content may be an important determinant in some tumor growth properties and in the response of tumors to radiation treatment. All 13 tumors studied were infiltrated with macrophages. However, individual tumor types varied greatly in their TAM content, showing a range of 9–83%. Both carcinomas and sarcomas contained, on average, about 40% macrophages. Wide variations in the TAM content of experimental animal tumors have been reported by others (5, 9, 14, 15, 22). TAM content was found to be 8–54% in chemically induced rat sarcomas (5), 9–54% (14) or 11–59% (22) in chemically induced murine sarcomas, and 4–61% in a variety of mouse and rat tumors of diverse origins (9). Of the 13 tumors we assessed, 12 were of spontaneous origin, and one (FSA) was a chemically induced tumor. A number of studies have assessed the TAM content of various human tumors, in which the percentage of TAM also showed a wide variation ranging from 0 to more than 50% (23–26). In general, however, the percentage of macrophages in human tumors was lower than that in experimental animal tumors.

Many factors are likely to be responsible for accumulation of macrophages in tumors. Tumor immunogenicity is one that has attracted much interest. Although earlier studies reported that immunogenic tumors showed greater infiltration by macrophages than nonimmunogenic or weakly immunogenic tumors (5, 15, 25), more recent studies on larger numbers of tumors failed to demonstrate a positive correlation between the 2 parameters (9, 22). It should be noted, however, that the earlier studies (5, 15) used rat tumors, whereas more recent studies have used mainly murine tumors (9, 22). Of the 13 tumors we studied, 2 sarcomas, FSA and FSA-II, and 3 carcinomas, MCA-29, MCA-K, and ACA-SG, were immunogenic.[5] The TAM content of these tumors was near or below the mean TAM content of all tumors assessed here. Thus, immunogenicity can be excluded as a major factor in the heavy infiltration of our tumors by macrophages.

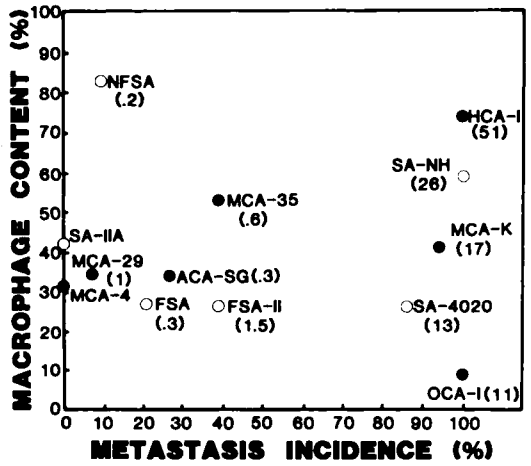

Fig. 4. Correlation between spontaneous propensity for metastasis of murine sarcomas (○) or carcinomas (●) and TAM content. Numbers in parentheses, mean number of lung metastases. Correlation coefficients are −0.16 for sarcomas, 0.14 for carcinomas, and −0.02 for all tumors tested.

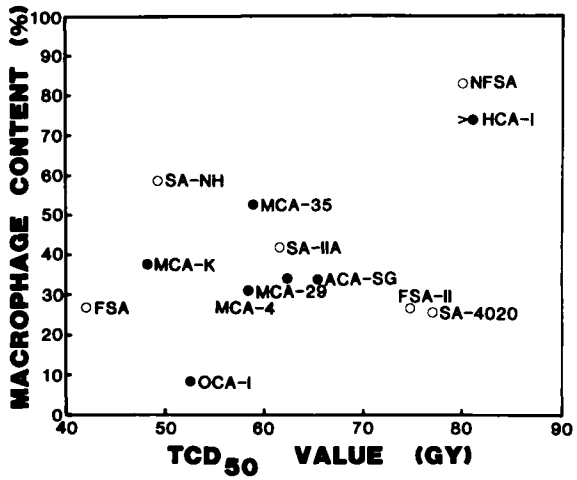

Fig. 5. Correlation between tumor radiocurability expressed in TCD_{50} values of murine sarcomas (○) or carcinomas (●) and TAM content. Correlation coefficients are 0.21 for sarcomas, 0.73 for carcinomas, and 0.43 for all tumors tested.

Table 4 *Effect of normal mouse peritoneal macrophages or TAM derived from NFSA tumor on in vitro growth of NFSA cells*

Four × 10^6 PE cells or the same number of TAM were plated, and 2 h later unattached cells were removed, and 10^5 NFSA cells added to macrophages. Two days later [^{3}H]thymidine (0.1 μCi/1 ml of medium) was added, and 1 h later the cells were harvested and counted for radioactivity.

Cells plated	[^{3}H]thymidine incorporation (cpm × 10^3)
NFSA	8.2 ± 0.8[a]
NFSA + PE macrophages	18.9 ± 0.9
NFSA + TAM	33.7 ± 1.1
PE macrophages	0.9 ± 0.1
TAM	5.1 ± 1.3

[a] Mean ± SE.

[5] J. Volpe and L. Milas, Unpublished observations.

Mice bearing 8-mm diameter solitary tumors had reduced numbers of macrophages in the peritoneal cavity (Table 2). This drop does not appear to be attributed to macrophage "consumption" by tumors, because the number of PE macrophages was not lower in mice bearing tumors with a higher TAM content. Because tumor cell products can inhibit various macrophage functions including chemotaxis, motility, and macrophage accumulation at inflammatory sites (27–29), it is reasonable to assume that they can influence the entry of macrophages into the peritoneal space.

A large body of evidence indicates that TAM interact with tumor cells and influence tumor cell growth. Macrophages from both experimental and human tumors can be cytostatic or cytotoxic to tumor cells or can even stimulate tumor cell growth. This study shows that TAM from NFSA were highly stimulatory to NFSA cell proliferation (Table 4). On the other hand, TAM from FSA have been shown to be cytotoxic to FSA cells (16). Although NFSA is nonimmunogenic (30), FSA possess a relatively strong immunogenicity (31). It is reasonable to assume that based on the TAM activity of FSA and NFSA, macrophages from other tumors investigated also are likely to interact with tumor cells from corresponding tumors.

A number of tumor growth characteristics were assessed to determine whether they correlated with the TAM content. The growth rate of already-established tumors showed no correlation with TAM, an observation similar to that reported by others (9, 32). Some investigators have reported a lack of correlation (32) or a positive correlation (9, 33) between TAM content and tumor latency period, *i.e.*, the time from tumor cell transplantation to generation of a palpable tumor. A positive correlation between these 2 parameters implies that TAM might be responsible for the delay in tumor take. To examine the relationship between TAM and tumor cell clonogenicity, we correlated TAM content with TD_{50} values (Fig. 1). The correlation was found to be significant and negative. Thus, fewer tumor cells were needed to generate s.c. tumors with tumor suspensions containing more macrophages, suggesting that the presence of a high percentage of macrophages is conducive to the survival of clonogenic tumor cells. The observed negative correlation between TAM content and the TD_{50} values and the lack of correlation between TAM content and the growth rate of established tumors imply that macrophages may play a role during the early establishing phase of tumor growth by promoting tumor cell proliferation. However, once tumors are established they no longer require help from TAM to maintain their growth rate.

The growth of solid tumors is often associated with necrosis, which develops primarily because of insufficient tumor vascularization. Because the removal of cellular debris is a major function of macrophages, one might expect macrophages to enter necrotic tumors in larger numbers to remove dead cells. However, little information is available on the association between the magnitude of necrosis and TAM content. Evans (34) stated that the degree of necrosis in several syngeneic murine and rat tumors had no influence on the proportion of macrophages. However, the data reported here show a significant negative correlation between the amount of necrosis and TAM content (Fig. 3). The statistical significance of this correlation is lost when the most necrotic tumor, OCA-I, is excluded from analysis, but even then a strong trend toward negative correlation remains. A number of explanations might account for this observation. The simple explanation is that necrosis may develop in tumors with insufficient TAM to remove dead cells or that under the influence of tumor cells TAM exhibit an impaired ability to ingest and process cellular debris. Although tumors release factors that inhibit various macrophage functions, including phagocytosis, TAM content appears to correlate well with phagocytic uptake and other functional and morphological criteria (9). However, a more plausible explanation is that tumors with a higher percentage of macrophages are better vascularized and, consequently, less necrotic. As macrophages produce angiogenic factors (35–37), a higher TAM content should provide a stronger angiogenic stimulus. We recently reported that the NFSA tumor, which has virtually no necrosis, does not exhibit the tumor bed effect phenomenon (38), *i.e.*, the reduced tumor growth rate in tissues exposed to ionizing radiation and considered to be a result of reduced vascular supply to tumors from a radiation-injured tumor bed (39, 40). We attribute this phenomenon, at least in part, to the strong angiogenic stimulus provided by NFSA's high TAM content (38). Also, relevant to this consideration is the observation that NFSA tumor cells secrete factors that stimulate bone marrow to increase macrophage production (41). Increased macrophage production would provide a larger cell pool as a source for TAM. Thus, in the case of NFSA, and possibly of other tumors with a high TAM content, a multifaceted active interaction may take place between tumor cells and macrophages, affecting both tumor cell proliferation and tumor angiogenesis. A high TAM content may thus promote tumor growth by direct stimulation of cell proliferation or by reducing cell loss from necrosis via angiogenic stimulation.

No correlation was found between TAM content and the ability of tumors to metastasize, a finding that is in accordance with other studies on this subject. As mentioned, earlier studies showed that tumors with a high percentage of macrophages were less metastatic and also more immunogenic (5, 6). A recent study by Talmadge *et al.* (9), in which a relatively large number of tumors was assessed, found no correlation between tumor immunogenicity and ability to metastasize. These results are in relatively good agreement with our findings: whereas of 5 immunogenic tumors 4 were moderately or poorly metastatic (FSA, FSA-II, MCA-29, and AC-SG) and one highly metastatic (MCA-K), of 8 nonimmunogenic tumors 4 were moderately or poorly metastatic (SA-IIA, NFSA, MCA-4, and MCA-35) and another 4 highly metastatic (SA-4020, SA-NH, OCA-I, and HCA-I). The absence of correlation between TAM content and metastatic ability does not mean that macrophages do not influence metastatic spread in individual tumor types, either by inhibiting or promoting it. The latter could be mediated by several mechanisms, including secretion of proteolytic enzymes that ease intravasation of tumor cells (42) and generation of metastatic cell variants through, for example, mutagenesis (43, 44). However, it should be noted that because a multitude of factors influence the metastatic behavior of tumors it is unreasonable to expect that TAM content or any other individual factor would show a good correlation with metastatic spread.

Also, it might be that the metastatic spread and other parameters of tumor progression are regulated more by the actual activation state of macrophages than by the extent of TAM infiltration. In this regard, Heppner *et al.* (44) and Loveless and Heppner (32) reported that macrophages from more metastatic tumors exhibited a higher activation state than those from less metastatic tumors. Activation status of macrophages, determined by the Fc receptor avidity, was assessed in 2 of 13 of our tumors (FSA and NFSA), and it was found that the relative proportion of activated macrophages was similar in both tumors (16). However, while FSA macrophages were

cytotoxic (16) NFSA macrophages were stimulatory (Table 4) to tumor cells from corresponding tumors.

Finally, we assessed whether tumor radiocurability by a single dose of γ-rays correlated with TAM content. The correlation was positive with tumor incurability but not significant for both sarcomas and carcinomas, particularly for carcinomas, in which the r value was 0.73. Thus, it appears that tumors tend to be more radioresistant when they contain a higher proportion of macrophages. One might expect the opposite simply on the basis that tumors with a high macrophage content contain fewer tumor cells that need to be eradicated. Tumor radioresponse is primarily determined by radiobiological factors such as total number of clonogenic cells, cell repair from radiation damage, cell repopulation and redistribution through the cell cycle, and tumor oxygenation (45). The evidence presented here and that reported by others (46–49) suggests that TAM activity may be an additional factor. Macrophages from the most radioresponsive sarcoma (FSA) were cytotoxic to FSA cells (16), whereas those from the most radioresistant sarcoma (NFSA) stimulated proliferation of NFSA cells (Table 4). Evans (46) reported that the growth of a murine fibrosarcoma in mice given whole-body irradiation was transiently inhibited, an event associated with a reduction in TAM content. This growth inhibition phase was followed by the resumption of a "normal" growth rate for this tumor and by the return of the TAM content to normal levels. The resumption of normal tumor growth was attributed to the stimulatory role of TAM on tumor cell proliferation (46). More recently, Evans *et al.* (49) showed that TAM stimulate the proliferation of murine tumor cells that survive treatment with cyclophosphamide. The obvious trend in our study toward an association between weak tumor radioresponse and a higher TAM content indicates that a high TAM content may indeed be deleterious to tumor radiotherapy. A significant negative correlation between TAM and TD_{50} values (Fig. 1) strongly suggests that TAM might promote the survival of tumor cells not killed by ionizing radiation.

Tumors with higher content of macrophages were found to be less necrotic (Fig. 3). One would anticipate that such tumors are also less hypoxic and consequently more radiocurable. Interestingly, 2 of our 13 (NFSA and HCA-1) tumors which were the least necrotic were the most radioresistant. Also, only 3 of our tumors (NFSA, FSA, and MCA-4) were analyzed for the extent of hypoxic cells when they were of comparable size; NFSA contained a significantly smaller fraction of hypoxic cells (50) than did FSA and MCA-4 tumors (10, 51). Thus, based on this limited information it is not possible to form reliable conclusions in regard to the relationship between the extent of macrophage content and hypoxic status of tumors, and the relationship of the latter to the TCD_{50} value. However, although limited, this information clearly points out that tumor radiocurability depends on a number of factors, including those reported in the present study.

Overall, our data show that murine sarcomas and carcinomas vary greatly in TAM content and that these macrophages might be active participants in the progression of malignant tumors. TAM content exhibited significant negative correlations with TD_{50} values and the extent of tumor necrosis and showed a strong trend toward positive correlation with reduced tumor radiocurability. As discussed, these correlations suggest that in the majority of the tumors assessed, TAM act as a conducive factor to proliferation of clonogenic tumor cells. However, we observed no correlation between TAM content and the growth rate of established tumors or metastatic spread.

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REFERENCES

1. Evans, R. Macrophages and neoplasms: new insights and their implication in tumor immunology. Cancer Metastasis Rev., *1:* 227–239, 1982.
2. Fidler, I. J. Macrophages and metastasis. A biological approach to cancer therapy: presidential address. Cancer Res., *45:* 4714–4726, 1985.
3. Milas, L., and Scott, M. T. Antitumor activity of *Corynebacterium parvum.* Adv. Cancer Res., *26:* 257–306, 1978.
4. Baldwin, R. W., and Pimm, M. W. BCG in tumor immunology. Adv. Cancer Res., *28:* 91–147, 1978.
5. Eccles, S. A., and Alexander, P. Macrophage content of tumors in relation to metastatic spread and host immune reaction. Nature (Lond.), *250:* 667–669, 1974.
6. Eccles, S. A. Macrophages and cancer. *In:* J. E. Castro (ed.), Immunological Aspects of Cancer, pp. 123–154. Lancaster, England: MTP Press, 1978.
7. Mantovani, A. Effects on *in vitro* tumor growth of murine macrophages isolated from sarcoma lines differing in immunogenicity and metastasizing capacity. Int. J. Cancer, *22:* 741–746, 1978.
8. Russell, S. W., and McIntosh, A. T. Macrophages isolated from regressing Moloney sarcomas are more cytotoxic than those recovered from progressing sarcomas. Nature (Lond.), *268:* 69–71, 1977.
9. Talmadge, J. E., Key, M., and Fidler, I. J. Macrophage content of metastatic and nonmetastatic rodent neoplasms. J. Immunol., *126:* 2245–2248, 1982.
10. Suit, H. D., and Suchato, D. Hyperbaric oxygen and radiotherapy of fibrosarcoma and squamous-cell carcinoma of C3H mice. Radiology, *89:* 713–719, 1967.
11. Volpe, J. P., Hunter, N., Basic, I., and Milas, L. Metastatic properties of murine sarcomas and carcinomas. I. Positive correlation with lung colonization and lack of correlation with s.c. tumor take. Clin. Exp. Metastasis, *3:* 281–294, 1985.
12. Milas, L., Hunter, N., Mason, K., and Withers, H. R. Immunological resistance to pulmonary metastases in C3Hf/Bu mice bearing syngeneic fibrosarcomas of different sizes. Cancer Res., *34:* 61–71, 1974.
13. Moore, K., and McBride, W. H. The activation state of macrophage subpopulations from a murine fibrosarcoma. Int. J. Cancer, *26:* 609–615, 1980.
14. Wood, G. W., and Gillespie, G. Y. Studies on the role of macrophages in regulation of growth and metastasis of murine chemically induced fibrosarcomas. Int. J. Cancer, *16:* 1022–1029, 1975.
15. Moore, K., and Moore, M. Intra-tumour host cells of transplanted rat neoplasms of different immunogenicity. Int. J. Cancer, *19:* 803–813, 1977.
16. McBride, W. H. Phenotype and functions of intratumoral macrophages. Biochim. Biophys. Acta, *865:* 27–41, 1986.
17. Peters, L. J., McBride, W. H., Mason, K. A., Hunter, N., Basic, I., and Milas, L. *In vivo* transfer of antitumor activity by peritoneal exudate cells from mice treated with *Corynebacterium parvum:* reduced effect in irradiated recipients. J. Natl. Cancer Inst., *59:* 881–887, 1977.
18. Camplejohn, R. S., and Penhaligon, M. The tumor bed effect: a cell kinetic and histological investigation of tumours growing in irradiated mouse skin. Br. J. Radiol., *58:* 443–451, 1985.
19. Finney, D. J. Quental response and the tolerance distribution. *In:* Statistical Methods in Biological Assay, pp. 454–456. London: Griffen, 1952.
20. Grdina, D. J., Basic, I., Mason, K. A., and Withers, H. R. Radiation response of clonogenic cell populations separated from a fibrosarcoma. Radiat. Res., *63:* 483–493, 1975.
21. Basic, I., Milas, L., Grdina, D. J., and Withers, H. R. *In vitro* destruction of tumor cells by macrophages from mice treated with *C. granulosum.* J. Natl. Cancer Inst., *55:* 589–596, 1975.
22. Evans, R., and Lawler, E. M. Macrophage content and immunogenicity of C57BL/6J and BALB/cByJ methylcholanthrene-induced sarcomas. Int. J. Cancer, *26:* 831–835, 1980.
23. Wood, G. W., and Gollahon, K. A. Detection and quantitation of macrophage infiltration into primary human tumors with the use of cell-surface markers. Int. J. Cancer, *59:* 1081–1087, 1977.
24. Svennevig, J. L., and Svaar, H. Content and distribution of macrophages and lymphocytes in solid malignant human tumors. Int. J. Cancer, *24:* 754–758, 1979.
25. Alexander, P., Eccles, S. A., and Gauci, C. L. L. The significance of macrophages in human and experimental tumors. Ann. NY Acad. Sci., *276:* 124–133, 1976.
26. Takeo, S., Yasumoto, K., Nagashima, A., Nakahashi, H., Sugimachi, K., and Nomoto, K. Role of tumor-associated macrophages in lung cancer. Cancer Res., *46:* 3179–3182, 1986.
27. Snyderman, R., and Pike, M. C. An inhibitor of macrophage chemotaxis produced by neoplasms. Science (Wash. DC), *192:* 370–372, 1976.
28. Rogan-Grgas, J., and Milas, L. Effect of tumor cell culture media and sera from tumor hosts on spreading, phagocytosis and antitumor cytotoxicity of *C. parvum*-activated macrophages. Cancer Immunol. Immunother., *6:* 169–173, 1979.
29. Norman, S. J., Schardt, M. C., and Sorkin, E. Macrophage inflammatory responses in rats and mice with autochthonous and transplanted tumors

induced by 3-methylcholanthrene. J. Natl. Cancer Inst., *72:* 175–184, 1984.
30. Ando, K., Hunter, N., and Peters, L. J. Immunological nonspecific enhancement of artificial lung metastases in tumor-bearing mice. Cancer Immunol., *6:* 151–156, 1979.
31. Suit, H. D., and Kastelan, A. Immunologic status of host and response of a methylcholanthrene-induced sarcoma to local X-irradiation. Cancer (Phila.), *26:* 232–238, 1970.
32. Loveless, S. E., and Heppner, G. H. Tumor-associated macrophages of mouse mammary tumors. 1. Differential cytotoxicity of macrophages from metastatic and nonmetastatic tumors. J. Immunol., *131:* 2074–2078, 1983.
33. Pross, H. F., and Kerbel, R. S. An assessment of intratumor phagocytic and surface marker-bearing cells in a series of autochthonous and early passaged chemically induced murine sarcomas. J. Natl. Cancer Inst., *57:* 1157–1167, 1976.
34. Evans, R. Macrophages in syngeneic animal tumors. Transplantation (Baltimore), *14:* 468–473, 1972.
35. Folkman, J. Tumor angiogenesis. Adv. Cancer Res., *19:* 331–358, 1974.
36. Polverini, P. J., Cotran, R. S., Gimbrone, M. A., Jr., and Unanne, E. R. Activated macrophages induce vascular proliferation. Nature (Lond.), *269:* 804–806, 1977.
37. Schor, A. M., and Schor, S. L. Tumor angiogenesis. J. Pathol., *141:* 385–413, 1983.
38. Milas, L., Ito, H., Hunter, N., Jones, S., and Peters, L. J. Retardation of tumor growth in mice caused by radiation-induced injury of tumor bed stroma: dependency on tumor type. Cancer Res., *46:* 723–727, 1986.
39. Hewitt, H. B., and Blake, E. R. The growth of transplanted murine tumors in preirradiated sites. Br. J. Cancer, *12:* 808–824, 1968.
40. Jirtle, R., Rankin, J. H. G., and Clifton, K. H. Effect of X-irradiation of tumour bed on tumour blood flow and vascular response to drugs. Br. J. Cancer, *37:* 1033–1038, 1978.
41. Milas, L., Faykus, M. H., Jr., McBride, W. H., Hunter, N., and Peters, L. J. Concomitant development of granulocytosis and enhancement of metastases formation in tumor-bearing mice. Clin. Exp. Metastasis, *2:* 181–190, 1984.
42. Weiss, L. Some mechanisms involved in cancer cell detachment by necrotic material. Int. J. Cancer, *22:* 196–203, 1978.
43. Weitzman, S. A., and Stossel, T. P. Mutation caused by human phagocytes. Science (Wash. DC), *212:* 546–547, 1981.
44. Heppner, G. H., Scott, E. L., Miller, F. R., Mahoney, K. H., and Fulton, A. M. Mammary tumor heterogeneity. *In:* G. L. Nicolson and L. Milas (eds.), Cancer Invasion and Metastasis: Biological and Therapeutic Aspects, pp. 209–221. New York: Raven Press, 1984.
45. Peters, L. J., Brock, W. A., Johnson, T., Meyn, R. E., Tofilon, P. J., and Milas, L. Potential methods for predicting tumor radiocurability. Int. J. Radiat. Oncol. Biol. Phys., *12:* 459–467, 1986.
46. Evans, R. Effect of X-irradiation on host-cell infiltration and growth of a murine fibrosarcoma. Br. J. Cancer, *35:* 557–566, 1977.
47. Stephens, T. C., Currie, G. A., and Peacock, J. H. Repopulation of γ-irradiated Lewis lung carcinoma by malignant cells and host macrophage progenitors. Br. J. Cancer, *38:* 573–582, 1978.
48. Currie, G. A. Promotion of fibrosarcoma cell growth by products of syngeneic host macrophages. Br. J. Cancer, *44:* 506–513, 1981.
49. Evans, R., Duffy, T., and Cullen, R. T. Tumor-associated macrophages stimulate the proliferation of murine tumor cells surviving treatment with the oncolytic cyclophosphamide analogue ASTA Z-7557: *In vivo* implications. Int. J. Cancer, *34:* 883–890, 1984.
50. Ando, K., Koike, S., Ikehira, H., Hayata, I., Shikita, M., and Ysaukawa, M. Increased radiosensitivity of a recurrent murine fibrosarcoma following radiotherapy. Gann, *76:* 99–103, 1985.
51. Stone, H. B., and Milas, L. Modification of radiation response of murine tumors by misonidazole (Ro 07-0582), host immune capability and *Corynebacterium parvum.* J. Natl. Cancer Inst., *60:* 887–893, 1978.