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Different Patterns of Macrophage Infiltration into Allogeneic-murine and Xenogeneic-human Neoplasms Growing in Nude Mice

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This study determined the distribution pattern of tumor-associated macrophages (TAM) in murine and human neoplasms growing subcutaneously in nude mice. Seven different human neoplasms (cancers of the breast, kidney, colon, prostate, lung, and skin, and a melanoma) and five different murine neoplasms (carcinomas of the lung, colon, and kidney, melanoma, and fibrosarcoma) were injected into nude mice. The murine tumors also were injected into syngeneic mice. Tumor-associated macrophages in small and large tumors were studied immunohistochemically by the use of several antibodies, including the macrophage-specific F4/80. The pattern of TAM distribution differed between mouse and human tumors. Regardless of histologic classification, TAM were uniformly distributed throughout all the murine neoplasms growing in syngeneic or nude mice. In the human neoplasms, TAM were found on the periphery of the lesions and in association with fibrous septae. The distribution of TAM in murine and human tumors was associated with a pattern of vascularization as determined by antibodies to basement membrane collagen type IV. Because the pattern of TAM distribution in neoplasms influences their antitumor activity, the data question the validity of the nude mouse model for the study of macrophage infiltration into human neoplasms. (Am J Pathol 1992, 141:1225–1236)

There is now a large body of evidence that activated macrophages play an important role in host defense against primary and metastatic cancers.[1] They become activated after contact with microorganisms or their products, such as endotoxin or cell wall skeleton, by interaction with lymphokines or by interaction with both signals.[1] Once activated, they can recognize and destroy neoplastic cells both *in vitro* and *in vivo* without injuring nontumorigenic cells,[2] discriminating between the two by a process that is independent of major histocompatibility antigens, tumor-specific antigens, cell cycle, or transformation phenotype.[2] Indeed, recognition is nonimmunologic, requiring cell-to-cell contact.[3,4] The systemic activation of macrophages by liposomes containing various immunomodulators has, for example, been shown to eradicate established metastases in both rodents[5–7] and dogs with autochthonous neoplasms.[9] Recent results of phase I clinical trials have concluded that this therapeutic approach is safe.[10,11]

To eradicate tumors, macrophages must infiltrate the lesions.[12–16] There is well-documented evidence that macrophages do infiltrate tumors,[17] and a recent immunohistochemical study of human tumors following an interleukin 2 immunotherapy regimen showed tumor-associated macrophages (TAM) in the regressing tumors.[18] Nevertheless, because it is not possible to obtain successive samples of human neoplasms from patients undergoing immunotherapy, the exact role of TAM in the growth or regression of human neoplasms remains unclear. Because many human tumors can be xenografted into immune incompetent mice, eg, nude mice,[19] we wished to develop this model for a kinetic study of TAM in human neoplasms. Before embarking on this ambitious study, we set out to determine the distribution and characteristics of TAM in human and rodent tumors growing in nude mice by immunohistochemical studies with a rat monoclonal antibody, F4/80, that is specific for mouse macrophages[20–22] and rabbit polyclonal antibodies MRP-8 and MRP-14 that identify inflammatory macrophages.[23,24] To correlate the distribution pattern of TAM with the pattern of tumor vascularization we used an an-

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tibody directed against basement membrane collagen type IV. To our surprise, the distribution pattern of TAM differed significantly between human tumors and their histologically matched murine tumors.

Methods

Animals

Specific pathogen-free BALB/c, C3H/HeN, C_{57}BL/6, and athymic Nu/Nu mice were purchased from the Animal Production Area of the National Cancer Institute (Frederick, MD). Animals were maintained in facilities approved by The American Association for Accreditation of Laboratory Animal Care, and in accordance with United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health regulations and standards.

Tumors

The human tumors used in this study were the A-375 melanoma, KM12C colon carcinoma, and its metastatic variant KM-12C-SM, A-431 epidermoid carcinoma, SN-12 renal cell carcinoma, H-226 lung carcinoma, PC-3 prostate carcinoma, and MDA-435 breast carcinoma.[19] The murine neoplasms used in this study were K-1735 melanoma and UV-2237 fibrosarcoma syngeneic to the C_3H/HeN mouse, CT-26 colon carcinoma, and RENCA renal cell carcinoma syngeneic to BALB/c mice, and 3-LL Lewis lung carcinoma syngeneic to C_{57}BL/6 mice.[25]

All tumor lines were grown as monolayer cultures in Eagle's minimal essential medium supplemented with 5% fetal bovine serum, vitamins, sodium pyruvate, L-glutamine, and nonessential amino acids. The complete medium was free of endotoxin as determined by the Limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. All cell cultures were free of mycoplasma, reovirus type 3, pneumonia virus of mice, K-virus, encephalitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD).

To produce tumors in syngeneic or nude mice, cultured cells (50% confluent) were given fresh medium 24 hours before harvest. The cells then were rinsed in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) and overlayed for 2 minutes with a 0.25% trypsin: 0.02% ethylenediaminetetra-acetic acid (EDTA) solution. The flask was tapped, and the cells were pipetted to produce a single-cell suspension. The cells then were washed in HBSS, and their viability was ascertained by trypan blue exclusion. Only suspensions with >95% viability were used for *in vivo* studies. The inoculum dose of cells differed among the different lines, consisting of the number of cells necessary to produce a tumor of 8 to 10 mm in diameter 4 to 6 weeks after subcutaneous implantation.

Immunohistochemical studies of each tumor line were carried out with at least three specimens (from three different mice). For each cell line, we also examined small (< 5 mm) and large (10 mm or more) tumors.

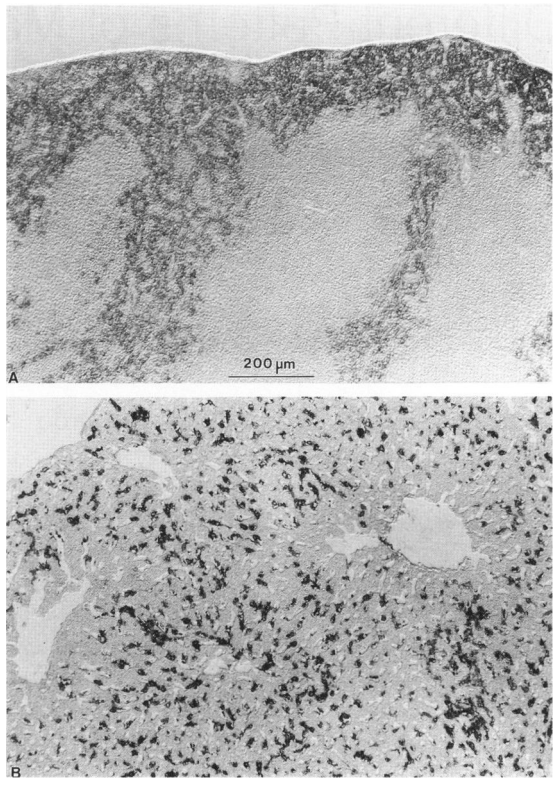
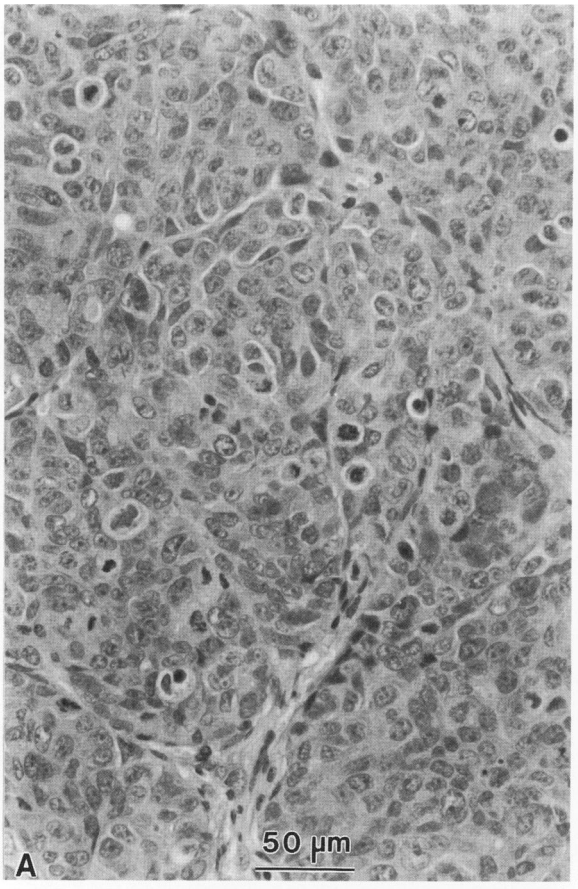


Figure 1. *F4/80 binds to macrophages in the red pulp of spleen* (**A**) *and Kupffer cells in the liver* (**B**). *F4/80-positive cells were rarely seen in the germinal centers of spleen.*

Antibodies

F4/80 is a rat monoclonal antibody that binds to a 160-kd plasma membrane glycoprotein present on mouse mononuclear phagocytes.[20] The hybridoma cells producing the antibody were the gift of Dr. David Hume (Uni-

Figure 2. *Light micrographs showing KM12 human* (**A,C**) *and CT-26 murine colon carcinomas* (**B,D**) *growing subcutaneously in nude mice. Hematoxylin and eosin-stained sections are shown in* (**A**) *and* (**B**), *and sections stained for F4/80 antigens are shown in* (**C–D**).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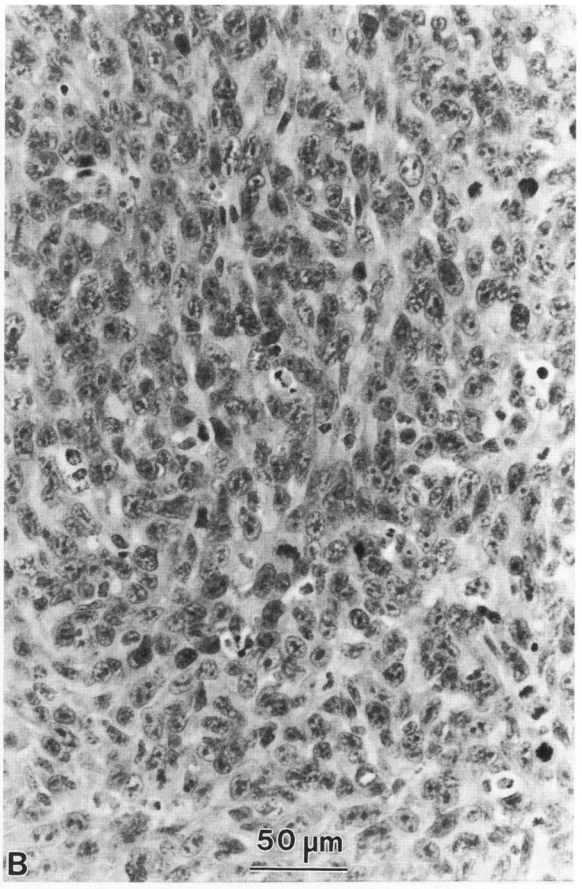
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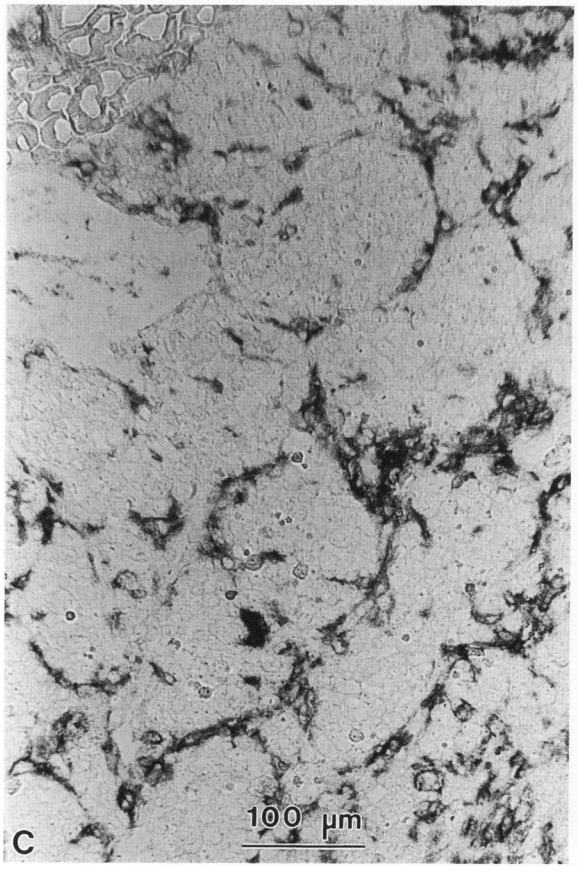
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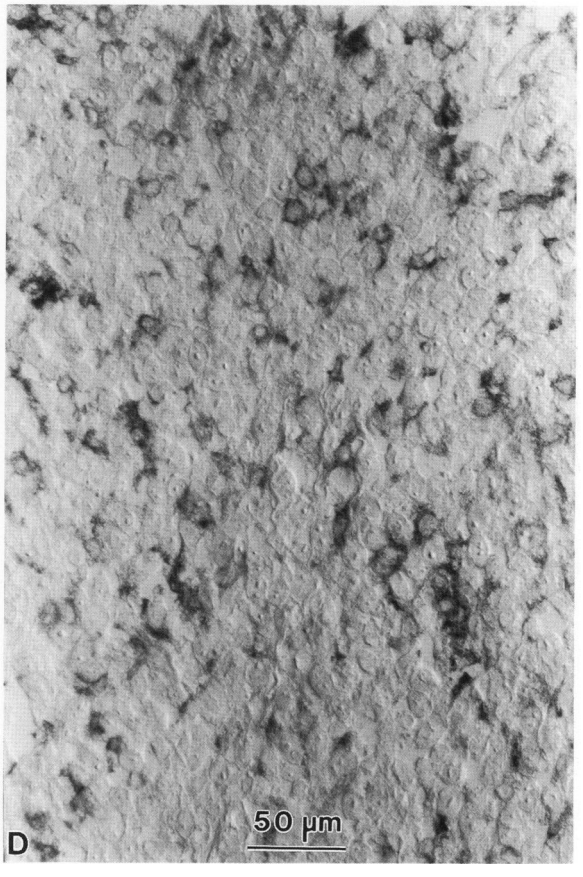
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versity of Queensland, Queensland, Australia). Rabbit polyclonal antibodies to the calcium-binding proteins MRP-8 and MRP-14 are selective markers for inflammatory macrophages[23,24]; they were the gift of Dr. Lajos Tarcsy (Ciba Geigy Ltd, Basel, Switzerland). Rabbit anti-mouse collagen type IV was purchased from Collaborative Research, Inc. (Bedford, MA). All immunogold antibodies and Silver Intense were made by Janssen Life Sciences (Amersham Corp., Arlington Heights, IL). MOMA was purchased from Bioproducts for Science, Inc., Indianapolis, IN. Anti-mac1, -mac2, and -mac3 were supernatants from hybridomas purchased from American Type Culture Collection (Rockville, MD).

Immunogold Labeling

The immunogold method was chosen in preference to immunoperoxidase because of difficulties encountered in removing endogenous peroxidase activity in some of the tissues. The second antibody controls used in the immunogold technique were undoubtedly cleaner than the immunoperoxidase controls. In addition, unlike the immunoperoxidase technique, the immunogold procedure does not use potentially carcinogenic substrates. Tumor tissues were cut into 5-mm pieces, placed in OCT compound (Miles Laboratories, Naperville, IL) in 1-inch aluminum caps, and snap frozen in liquid nitrogen. The resulting blocks were stored in a −80°C freezer until ready to cut. Sections (8 to 10 μ) were cut in an IEC Minotome cryostat (International Equipment Co., MA), air dried for 30 minutes, and fixed in either 0.125% glutaraldehyde in phosphate-buffered saline (PBS) for 10 minutes or cold acetone (Fisher certified). Other types of acetone that we tested gave unsatisfactory results. The slides were washed three times with PBS, the surface around the tissue was wiped dry, and a circle around the tissue was drawn with a PAP pen (Kiyota International Inc., Arlington Heights, IL) to confine the incubation fluid to the tissue area. All incubations were done in a humidified chamber at ambient temperature. Immunocytochemical detection of F4/80 was achieved by sequential incubation of the tissue with 1% normal goat serum plus 1% bovine serum albumin in PBS for 20 minutes followed by incubation with the primary antibody for 1 hour, extensive washing with PBS, and incubation with gold-labeled secondary antibody for 1 hour. The samples then were washed with PBS, fixed with 2% glutaraldehyde in PBS for 10 minutes, and washed with distilled water three times. To minimize background staining, the samples were incubated with Silver Intense twice, 5 minutes each time. This procedure gives clean negative controls consistently. The samples were examined as wet mounts using glycerol/PBS as the mounting medium or were permanently mounted using Crystal Mount (Biomeda, Fisher Scientific).

Spleens and livers from normal mice were used as positive controls for F4/80 antibody, and a BCG granuloma induced in mouse skin was used as positive control for anti-MRP8 and MRP14. Antibody control consisted of incubation of adjacent sections with normal goat serum followed by the secondary antibody.

Enumeration of macrophages, where feasible, was made before application of a counterstain to the sections. To clearly illustrate the different patterns observed, the photomicrographs shown in this report were obtained from sections that were not counterstained. The sections were examined using Nomarski optics to obtain detail in sections that were not counterstained, and in cases of

Table 1. *Distribution of F4/80 Positive Cells in Murine and Human Tumors* Grown Subcutaneously in Athymic Nude Mice*

	Distribution of F4/80-positive cells		
	Peritumoral	Intertumoral	Intratumoral
Mouse tumors			
K-1735†			+ + +
UV-2237†			+ + +
3LL			+ + +
CT26†			+ + +
RENCA		+ +	+ +
Human tumors			
A375	+ + +	+ +	+
KM12SM	+ + +	+ +	+/−
KM12C	+ + +	+ +	+/−
A431	+ + +	+ +	+/−
SN12	+ + +	+ +	+
PC3	+ + +	+ +	+
H226	+ + +	+ +	+
MDA-435	+ + +	+ +	+

* All tumors were about 0.5 cm in diameter.
† These tumors were also injected subcutaneously into syngeneic mice.

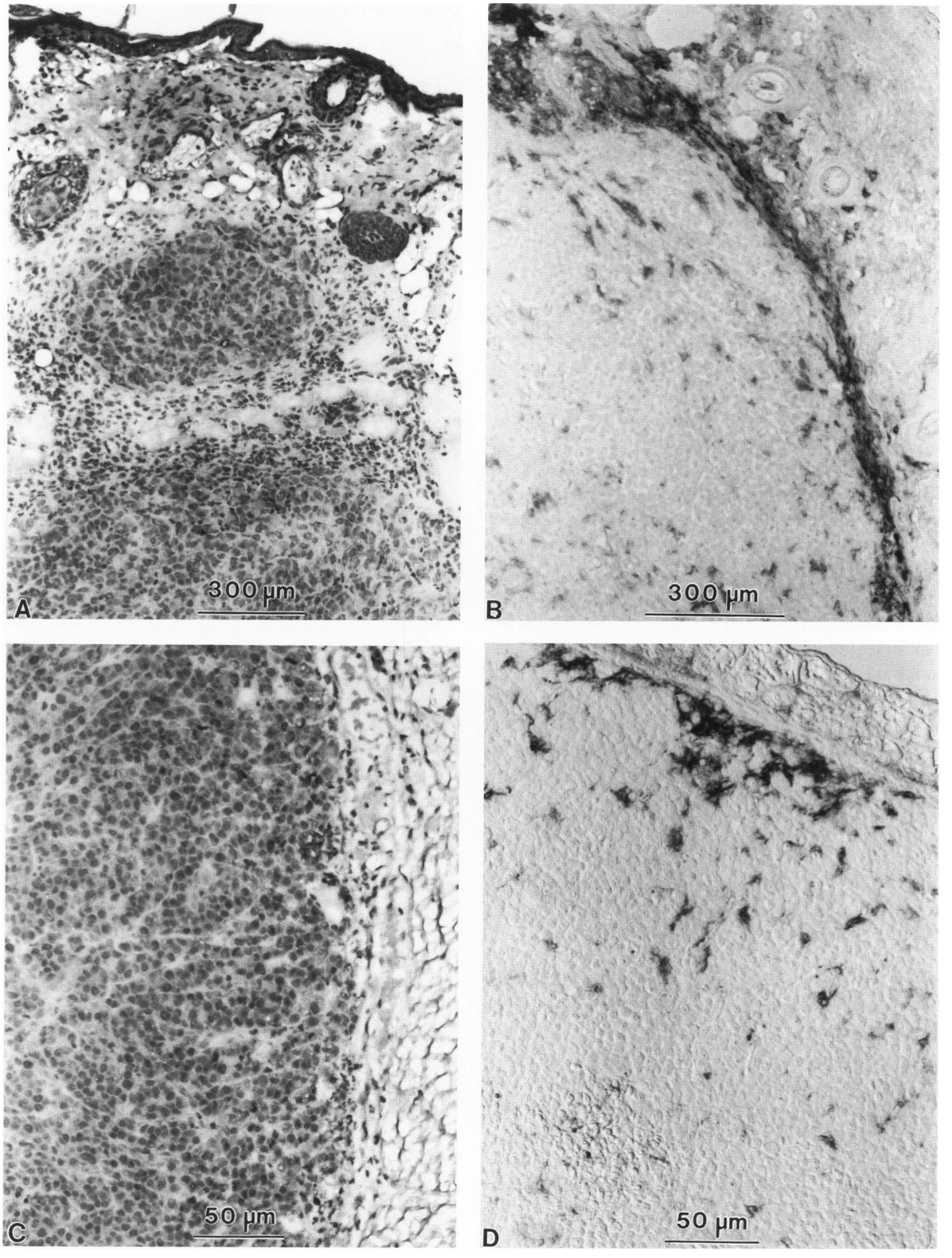


Figure 3. *Human mammary carcinoma exhibited a predominantly peritumoral distribution of F4/80 cells with few intratumoral F4/80 positive cells.* (A) *and* (C) *are H & E-stained sections, and* (B) *and* (D) *are F4/80-stained sections. Note the peritumoral distribution of F4/80-positive cells and few intratumoral macrophages.*

melanotic melanoma, the samples were examined using epipolarization optics to distinguish melanin pigment (which thereby becomes invisible) from the reaction product (in bright field microscopy, the two are indistinguishable). Epipolarization microscopy was performed in a Nikon Optiphot equipped with a filter cube and a 50-W HBO mercury lamp.

Morphometric Estimation of the Areal Fraction Occupied by F4/80+ Areas in Tumor Tissue

F4/80+ cells are distributed throughout the murine tumors and in distinct sectors in human tumors. The label is in good contrast relative to the background; thus, it is possible to quantify the areal fraction of tumor tissue that contains F4/80-positive cells. The F4/80-positive areas would represent profiles of cross-sections of cells or cytoplasmic processes. Five random fields were examined carefully so as not to include the peritumoral region of the tumor, where the F4/80-positive cells are usually high. Each field had an area equivalent to 0.1513 mm^2. An electronic grid slightly smaller than the area being examined was overlaid on the image of the section, and only profiles that fell within the grid and those touching the left and lower borders of the grid were enumerated. Sections obtained from different portions of the tumor exhibited a uniform pattern of macrophage infiltration; thus, we are confident that the analysis done in a given section is representative of the tumor being tested. Image analysis was performed in an IBAS image analyzer (Carl Zeiss, Inc., Thornwood, NY).

Results

Macrophages in Spleen and Liver

In the first set of studies we determined the validity of using F4/80 antibodies to identify macrophages in the spleen and liver. F4/80 antibody bound to the Kupffer cells in the liver of nude mice and to mononuclear phagocytes in the red pulp of the spleen (Figure 1). The distribution of macrophages in these samples was also confirmed using other anti-macrophage sera such as MOMA, as well as anti-macl, anti-mac2, and anti-mac3. Because F4/80 produced the most consistent and specific labeling, it was used routinely in this study. Macrophages in a subcutaneous BCG granuloma were strongly positive for anti-MRP14 and anti-MRP8 antibodies, confirming published reports.[23,24]

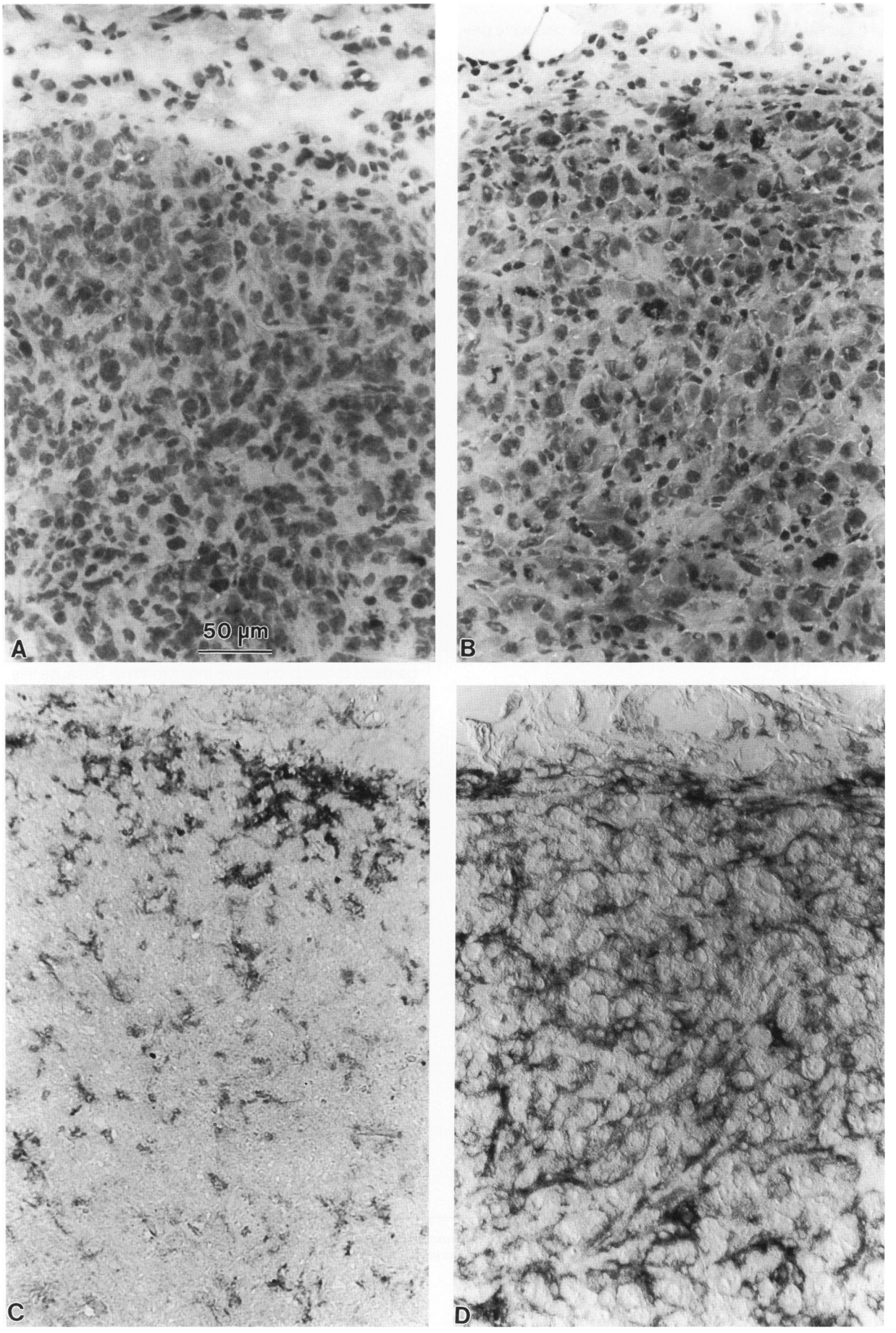
Pattern of Macrophage Distribution in Mouse Neoplasms

In the next set of studies, we injected human colon carcinoma KM12 (Figure 2A) and mouse colon carcinoma CT-26 (Figure 2B) into the subcutis of nude mice. The distribution of F4/80-positive cells in subcutaneous tumors was scored as peritumoral when positive cells were confined to the area surrounding the tumor. Most of these F4/80-positive cells were not contiguous with tumor cells at the periphery. F4/80-positive cells that were associated with connective tissues but were not directly associated with tumor cells were considered intertumoral (Figure 2C). F4/80-positive cells found mixed with tumor cells were considered intratumoral (Figure 2D). The data summarizing the pattern of macrophage distribution in subcutaneous tumors are shown in Table 1. Five mouse tumors (melanoma, fibrosarcoma, lung carcinoma, renal carcinoma, colon carcinoma) were injected subcutaneously into nude mice or into syngeneic mice. Regardless of the recipient animal, both small (5 mm) and large (> 10 mm) tumors exhibited an intratumoral distribution pattern of F4/80-positive macrophages, ie, the TAM were distributed throughout the progressively growing neoplasms.

Pattern of Macrophage Distribution in Human Neoplasms

We next examined the pattern of nude mouse macrophage infiltration into seven different human tumors (melanoma, carcinomas of the skin, kidney, prostate colon, breast, and lung) with histologies similar to that of the murine neoplasms. Regardless of histology, the pattern of macrophage infiltration differed from that found for murine neoplasms. In all human neoplasms, F4/80-positive cells were primarily peritumoral or intertumoral, ie, in the periphery of the tumors or associated with connective tissue septae (Table 1). Human colon and breast carcinoma showed large tumor islands, and F4/80-positive cells were confined to the peritumoral and intertumoral regions of the tumor, thus presenting a lobular appearance (Figures 2C, 3A–D). A similar pattern of macrophage infiltration was observed in a human colon carcinoma injected subcutaneously into SCID mice. In renal carcinoma, the tumor islands were considerably smaller

Figure 4. *Human renal carcinoma, SN12, exhibited a predominantly peritumoral distribution of F4/80+ cells* **(A,C)**. *In contrast, mouse renal carcinoma RENCA* **(B,D)** *show F4/80-positive cells throughout the tumor. The F4/80 cells appeared to form a network with tumor cells trapped inside the network.*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(Figure 4A, C); thus, even if F4/80-positive cells were predominantly in the peritumoral and intertumoral regions, they appeared randomly distributed in the tumors (Figure 4C). In mouse renal carcinoma, RENCA, F4/80-positive cells were seen intratumorally (Figure 4B, D).

Detailed Immunohistochemistry

Because we examined a large number of specimens with similar results, the presentation of detailed histologic data will be restricted to only a few examples. A clear difference in the distribution of TAM between mouse CT-26 colon cancer and human KM12 colon cancer is shown in Figures 2 and 3. Additional examples for the unique peripheral-peritumoral distribution of F4/80-positive TAM in human tumors growing in nude mice are shown for renal cell carcinoma (Figure 4B) and breast carcinoma (Figure 3B, D).

In murine tumors, F4/80-positive cells were seen either as dendritic cells whose processes could be found between tumor cells or as long spindle cells with processes that appeared to form a network (Figure 4D).

Staining of the above tumors with antibodies to MRP14 and MRP8 showed very few positive cells in the peritumoral area.

Macrophage Distribution and Tumor Vascularity

To determine whether the pattern of F4/80-positive cell distribution in tumors was related to their pattern of vascularization, mouse and human tumors were stained with an antibody to collagen type IV that can identify blood vessels. In both the human colon carcinoma and renal carcinoma, the blood vessels were stained in the periphery and connective tissue septae but not within the tumor tissue itself, suggesting a lobular architecture (Figure 5A–D). In contrast, mouse colon carcinoma growing subcutaneously in nude mice exhibited many small blood vessels in the periphery and throughout the tumor (Figure 5E, F).

Morphometric Analysis of F4/80-positive Areas in Murine and Human Tumors

To compare the F4/80 positivity between tumors, we analyzed by image analysis the amount of F4/80 labeling in sections of three mouse tumors and three human tumors implanted in nude mice. F4/80-positive profiles and areal fraction were determined, and in all cases, the amount of F4/80 labeling in murine tumors was two to three times higher than that observed in human tumors (Table 2).

Discussion

The current study was undertaken to evaluate the validity of the nude mouse xenograft system as a model for examining macrophage infiltration into tumors of different histologies and origins. Seven human neoplasms were studied, and five mouse neoplasms of matching histologic classification were used as controls. Regardless of whether their histology matched, the pattern of macrophage distribution differed between human and mouse tumors, raising doubts about the validity of this model for human neoplasms.

The distribution pattern of host inflammatory cells into tumors has been studied for many years. In regressing mouse Moloney sarcomas, TAM were found throughout the tumors,[16] whereas in progressive mouse Moloney sarcomas, TAM were confined to the periphery of the tumor or accompanying bands of connective tissues.[16] Our data, derived from five different mouse tumors, suggest that TAM are distributed throughout the tumors regardless of how progressive their growth is. Because this pattern was found in both nude mice and syngeneic mice, it could not be attributed to the immunogenicity of the neoplasms.

Talmadge et al.[26] found that murine tumors with a high macrophage content required a longer induction period from injection of the cells into animals until production of a palpable tumor.[26] These results were corroborated in a study by Pross and Kerbel,[27] who studied methylcholanthrene-induced murine sarcomas and found that tumors with a low level of macrophage infiltration appeared more rapidly than tumors with a high level of macrophage infiltration. Both of these studies also found that there was no correlation between macrophage content and *in vivo* growth rate (after tumors were palpable) or *in vitro* growth rate. The primary conclusion of these studies, as well as one by Evans and Lawler,[28] was that accumulation of mononuclear phagocytes differs among different tumors and does not necessarily correlate with tumor cell immunogenicity or metastatic properties.

Normann reviewed studies with autochthonous and transplanted tumors and summarized the mechanisms by which macrophages accumulate within tumors in four

Figure 5. *Vascularity of different tumors was demonstrated by immunostaining with anti-collagen Type IV, KM12* **(A–B)**, *SN12* **(C–D)**, *CT26* **(E–F)**. *Note the lobular pattern in KM12 as opposed to the diffuse staining reaction observed in CT26.*

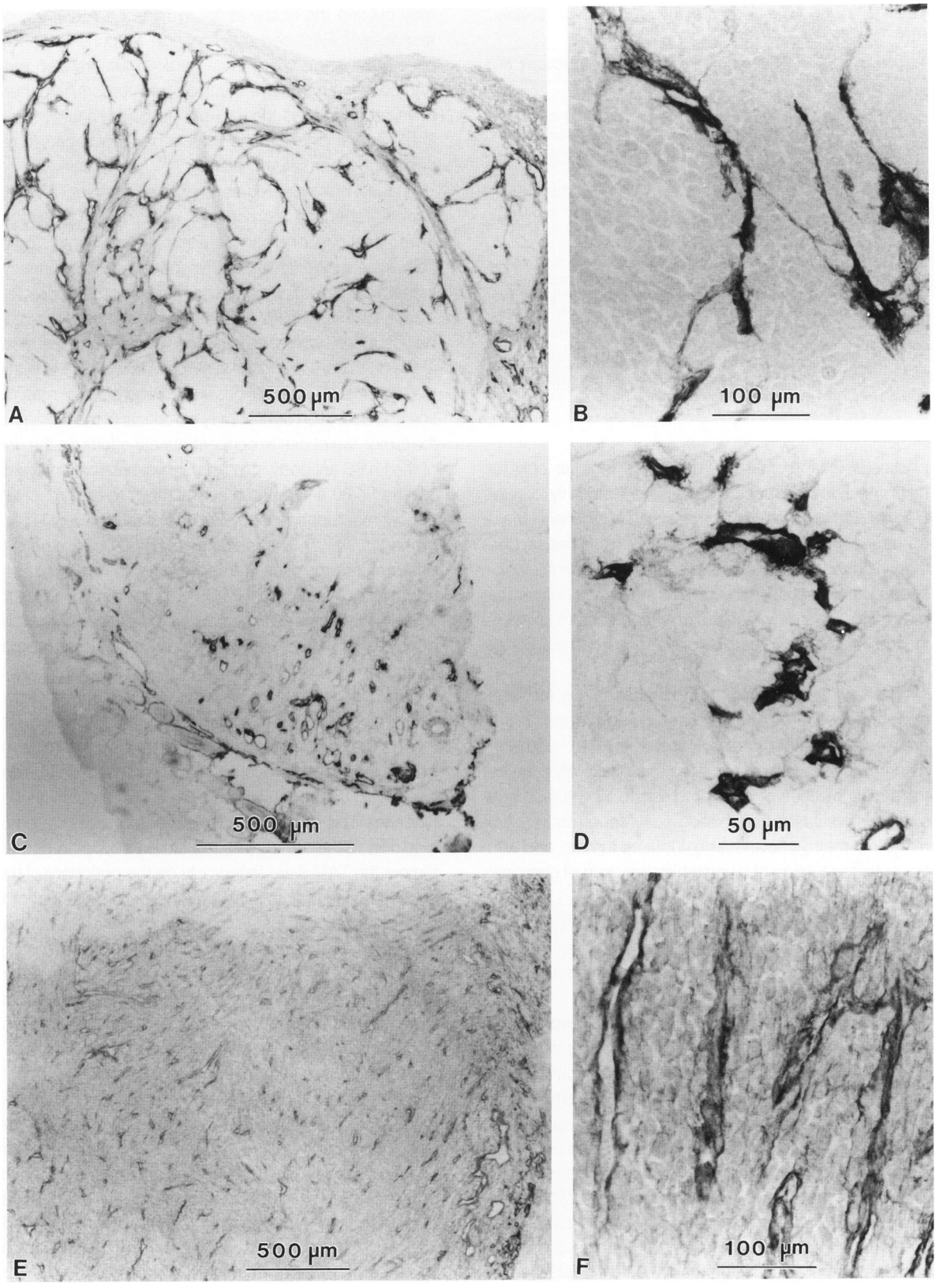
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Table 2. *F4/80 Content of Murine and Human Tumors Injected into Nude Mice*

Tumor	No. of profiles*	Areal fraction† (%)
Mouse tumors		
K-1735	177 ± 16‡	10.84 ± 1.06
CT26	149 ± 8	10.71 ± 0.42
RENCA	147 ± 7	12.25 ± 0.69
Human tumors		
A375	85 ± 16	5.72 ± 0.65
KM12	55 ± 7	5.34 ± 3.07
SN12	75 ± 26	4.26 ± 1.91

* Because a histological section only shows a two-dimensional view of a portion of the tumor, F4/80 positivity is expressed as either number of profiles per unit area or areal fraction of the tumor. Profiles refer to areas of F4/80 positivity regardless of size and/or location of cells; thus, some profiles may contain two or more cells or only cytoplasmic portions of cells.

† Areal fraction refers to the fraction of the field that is occupied by F4/80 positive profiles, i.e., Areal fraction = Total area of F4/80 positive profiles/Area of specimen examined × 100.

‡ Means value ± SD from 0.1513 mm^2 areas.

phases.[12] During phase 1, described thus far only for micrometastases, macrophages are randomly distributed throughout the tumor in relatively high density. Macrophages appear to accumulate freely in this phase, during which there is no indication that the inflammatory response is impaired. This suggests that *in situ* carcinoma may have a relatively high macrophage content, explaining the inflammatory reaction often seen in early carcinomas.[12]

In phase 2, tumor cell growth outpaces macrophage accumulation. Therefore, macrophage density decreases. In separate studies, Bugelski et al[29–31] and Normann et al[32] have demonstrated this phase after the intravenous injection of highly metastatic lung tumor variants, in metastases from histiocytic lymphomas in SJL/J mice, in 3-methylcholanthrene–induced tumors, and with transplanted tumors of diverse histology and of chemical or viral origin.

Phase 3 of macrophage infiltration into tumors corresponds to the midpoint of the clinical course of most tumors. In this phase, macrophage accumulation keeps up with the logarithmic growth of tumor cells.[32]

The later clinical course of tumors corresponds to phase 4 of macrophage accumulation. At this point, the tumor cells continue to multiply while the number of macrophages infiltrating the tumor decreases, causing an impairment of inflammatory responses in these advanced tumors.[33–40] Bugelski et al found that the ratio of macrophages to tumor cells within a nonimmunogenic rodent tumor falls dramatically with increasing size of the tumor and progressive metastatic growth[39,40] and concluded that the macrophage content of large tumors is not proportional to that of small tumors or micrometastases.[39]

Several investigators have suggested possible mechanisms for the decreased recruitment of macrophages into larger tumors. These large tumors and metastases may outstrip the host's ability to mount a macrophage response.[41] Reduced macrophage responsiveness or effects of the geometry of large solid tumors also may decrease macrophage invasion into tumors.[41] Others have demonstrated that soluble products of tumor cells from several different cell lines impair macrophage recruitment in normal animals.[42]

In most previous studies, characterization of TAM was accomplished by morphologic examination, histologic frozen sections, or disaggregation of tumors into single-cell suspension followed by histochemical or immunocytochemical detection. Although TAM can be characterized in cell suspensions,[42] the isolation procedure may be associated with a selective loss of cells, and the technique does not allow for precise localization of TAM within the tumor. Because the localization and state of maturation and activation of TAM may be critical to the biology of the tumor, we chose to concentrate our efforts on the use of the F4/80 antibody in immunohistochemical analyses.

The F4/80 antigen has been shown to be present in mature macrophages, but it does not recognize all macrophages.[15,20–22] The presence of F4/80-positive cells in different organs was demonstrated by immunocytochemical detection of F4/80[21] proteins; MRP8 and MRP14, which are selective markers for inflammatory phagocytes,[23,24] also were used to identify host macrophages.

The different pattern of distribution of F4/80-positive cells in the human and murine tumors is intriguing. Although the murine tumors exhibited a very high macrophage content and macrophages were intimately associated with tumor cells, the growth of the tumors was not hindered. This suggests that the macrophages were not cytotoxic to the tumor cells. As stated above, in human tumors the TAM were found only on the periphery of the subcutaneous tumors. One explanation for this phenomenon may be that human tumors growing at an ectopic site are often encapsulated.[19] Macrophages are closely associated with the formation of a fibrous capsule surrounding growth-aggregated or regressing mouse tumors.[43] Murine macrophages were also shown to produce (among many proteins) collagen type I.[44]

Activated macrophages produce a variety of growth factors,[45] including mitogens for endothelial cells.[46] The distribution of blood vessels in the human tumors studied here differed from that revealed in the murine tumors. In the former, the vessels surrounded the tumor growing as nodules, whereas in murine neoplasms the vessels were more homogeneously distributed throughout the neoplasms. Although this blood vessel distribution did not correlate with the growth pattern of the tumors, it did correlate with the pattern of TAM distribution. This observation supports the suggestion of Evans[47] that in the tumor tissue "the number of macrophages extravasating capil-

laries is proportional to the extent of vascularization and the permeability of vessels."

In summary, the pattern of TAM distribution into most human neoplasms growing in nude mice is very different from that of mouse neoplasms. The distribution of macrophages in a large number of autochthonous human neoplasms was reported by Wood and Gollahon[48] and Svennevig and Svaar[49] using the EA rosetting assay and nonspecific esterase assay, respectively. Most human tumors studied exhibited peritumoral and intertumoral distribution of lymphocytes and macrophages. This reported pattern of macrophage infiltration was also apparent in our study of human tumors growing as xenografts in nude mice.

Acknowledgments

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