A Gelatin Sponge Model for Studying Tumor Growth: Flow Cytometric Analysis and Quantitation of Leukocytes and Tumor Cells in the EMT6 Mouse Tumor

Emmanuel T. Akporiaye,² Sigrid J. Stewart, Anita P. Stevenson, and Carleton C. Stewart³

Experimental Pathology Group, Life Sciences Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545

ABSTRACT

This study examined the recruitment of host cells into a progressing EMT6 tumor following the inoculation of tumorigenic cells into a preimplanted gelatin sponge. Tumor cell proliferation occurred to a greater extent in sponge tumors than in tumors obtained by direct subcutaneous injection of tumor cells. Blank sponges, lacking tumorigenic cells and used as controls, elicited an inflammatory response characterized by a modest infiltration of granulocytes and macrophages whose numbers, after Day 7 postimplantation, remained unchanged for 21 days of sponge residence in the animal. In contrast, when the sponge contained tumor cells, there was a continuous increase in host cell infiltration that paralleled the increase in tumor cells. Whether in a sponge or not, tumor cells represented more than half of the recovered cells by Day 21 after tumor cell inoculation. Macrophages comprised the greatest fraction of host cells infiltrating the tumors. Flow cytometric analysis and morphological examination of disaggregated tumors indicated that macrophages (19-51%) made up the largest proportion of host cells followed in order by granulocytes (6-18%) and lymphocytes (2-9%). Sorting of marker-labeled cells revealed that 94% of the surface immunoglobulin-bearing cells were macrophages. Twenty-two % of the cells bearing the Thy1.2 marker were lymphocytes, and 68% were macrophages.

The data confirm the occurrence of a cellular host response in the tumor-bearing animal which is distinct from the foreign body reaction elicited by a blank sponge. Additionally, the sponge system described here represents a recoverable environment that would facilitate the study of in vivo host-tumor cell interactions that occur during early tumor development or later during therapy-induced tumor rejection when a palpable tumor is not present.

INTRODUCTION

Studies designed to examine the host immune response to transplantable or autotnchonous solid tumors have used s.c. inoculation as a route for introducing tumorigenic single cell suspensions into the test animal. The potential for loss of injected cells from the inoculation site by this method and the subsequent reduction in initial tumor cell burden led to the utilization of sponge matrices to provide anchorage for injected cells (1, 2). In addition to acting as a receptacle for injected cells, the sponge-matrix retains infiltrating host cells. The physical attribute of retaining host and tumor cell components makes the sponge system a suitable model for studying in vivo host-tumor interactions in a recoverable environment. Roberts and Häyry (3) first implanted urethane sponges coated with peritoneal cells into an allogeneic host for the purpose of retrieving allotransplant effector cells. Lymphocytes infiltrating the sponge allotransplant were successfully recovered by gentle compression of the sponge and were demonstrated to be cytotoxic in vitro towards donor cells.

Thymus-derived lymphocytes (T-cells) with tumoricidal activity have been similarly recovered from preimplanted sponges injected with the Moloney virus-induced murine sarcoma (4). More recently, a preimplanted gelatin sponge matrix for the inoculation of tumor cells has been described (1). In this system, host and tumor cells can be recovered by digestion of the sponge in collagenase solution. In addition to the high yield and ease of cell recovery, the sponge model provides a means to study some of the complex host-tumor cell interactions occurring, especially in the early stages of tumor progression or the late stages of immunological or therapy-induced rejection when a palpable tumor nodule is not discernible. In order to validate the use of the sponge model for the aforementioned studies, it was considered necessary to first of all address the issue of whether the sponge microenvironment affects the degree of host cell accumulation and tumor cell proliferation.

The study described in this paper compared the kinetics of host cell infiltration and tumor cell proliferation in the EMT6 carcinoma after inoculation into syngeneic BALB/c mice DSC⁴ or into a 2-day preimplanted gelatin sponge. In this paper, we also describe a method that permits the resolution of tumor cells from host cells in the EMT6 tumor by flow cytometry and cell sorting based on their differential DNA content and labeling with fluoresceinated antibodies. We present evidence that demonstrates a progressive increase in intratumor macrophages, lymphocytes, and granulocytes with tumor age. The recruitment of these host cells occurs to a greater extent in sponge tumors than in the tumors which resulted from direct s.c. inoculation of tumor cells. Blank sponges lacking tumor cells elicited a foreign body reaction characterized by a smaller, yet significant, accumulation of host cells. The results also suggest that tumor cell proliferation occurs at a slightly faster pace in sponge tumors than in DSC tumors.

⁴ The abbreviations used are: DSC, direct subcutaneous injection; AO, acridine orange; PI, propidium iodide; PBS, calcium- and magnesium-free Dulbecco’s phosphate-buffered saline; MOPS, 2 N-[morpholinopropane sulfonic acid; FITC, fluorescein isothiocyanate; FCM, fluorescence activated cell sorter; FGAR, fluoresceinated goat anti-rat immunoglobulin; HO-PAB, phosphate-buffered saline containing 0.5% bovine serum albumin, 0.1% sodium azide, and Hoechst 33342 (2 μg/ml); FC5, flow cytometry; slg, surface immunoglobulin; MAb, monoclonal antibody.

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² Present address: Department of Biology, Northern Arizona University, Flagstaff, AZ 86011.
³ To whom requests for reprints should be addressed.

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MATERIALS AND METHODS

**Mice.** Eight- to 12-wk-old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were used in this study. The animals were housed in the Los Alamos animal care facility and fed *ad libitum*.

**Tumor.** The EMT6 tumorigenic cell line was obtained originally from Dr. Sara Rockwell (5). The EMT6 cell line displays near tetraploid DNA content and is a subline of an early passage of the KHJJ line that was derived from a primary mammary carcinoma arising in a BALB/c mouse (5). The cells were maintained and propagated in vitro by serial passage in alpha-minimal essential medium supplemented with 10% fetal bovine serum.

**Sponge Implantation and Injection of Tumor Cells.** Sterile gelatin sponges (Spongostan, Ferrosan, Denmark), each measuring approximately 17 x 18 x 10 mm, were surgically implanted s.c. to the neck of pentobarbital sodium (60 mg/kg body weight)-anesthetized animals as previously described (1). The backs of the mice were shaved free of hair and thoroughly swabbed with 70% ethanol before sponge implantations were performed. Sponges were implanted 2 days prior to tumor cell injection.

Subconfluent, exponentially growing EMT6 monolayer cells were detached with trypsin (0.25% trypsin, 5 min at 37°C) and adjusted to a concentration of 1 x 10⁶ cells/ml in sterile saline solution. Animals bearing sponges were each given injections of 1 x 10⁵ viable cells in 0.1 ml through the skin and into the sponge. Animals not harboring sponges also received 1 x 10⁵ cells s.c. injected through the skin. Animals implanted with blank sponges did not receive tumor cells.

**Tumor Retrieval and Disaggregation.** Tumor sponges, blank sponges, and tumors which resulted from direct s.c. inoculation of tumor cell suspensions were surgically removed at selected intervals from animals sacrificed by CO₂ overdose (dry ice). Tumor disaggregation was accomplished by a modification of the method described by Kerkoff (6) for thyroid gland dissociation. The predissociated enzyme cocktail contained 25 ml of saline G (composition in g/l: glucose, 1.1 g; NaCl, 8.0 g; KCl, 0.4 g; Na₂HPO₄·7H₂O, 0.29 g; KH₂PO₄, 0.15 g; phenol red, 0.005 g; MgSO₄·7H₂O, 0.15 g; CaCl₂·2H₂O, 0.016 g) containing 50 mg of collagenase (200 units/mg; Worthington Biochemicals, Freehold, NJ) and 500 mg of bovine serum albumin (Fraction V; Sigma, St. Louis, MO). Tumor explants were minced with a pair of scissors in a 10-ml beaker. Minced tumors were immediately resuspended in 5 ml of the enzyme mixture and transferred with a 10-ml pipet into 100-mm spinner culture flasks (Bellco, Vineland, NJ). Enzyme solution was added to bring the total volume in each flask to 25 ml. Spinner culture flasks were incubated at 37°C for 2 h with constant stirring. The resulting cell suspension was sequentially filtered through 380-μm and 140-μm wire screens. All manipulations were performed under sterile conditions. Complete disaggregation of blank sponges, DSC, or sponge tumors was achieved after digestion in collagenase solution for 2 h.

**Determination of Cell Number and Viability.** The viability and yield of the dispersed cell suspension were determined by counting the cells in a hemocytometer after staining with PI and AO using a fluorescence microscope (mercury arc) with an exciter filter (BP 450-490), a chromatic beam splitter (FT 510), and a barrier filter (LP 510) (7). A 100-μl aliquot of cell suspension was added to 10 μl of PI (200 μg/ml in PBS) and 100 μl of AO (10 μg/ml in PBS). Viable cells are stained with AO (green nuclear fluorescence), and dead cells are stained with PI (red nuclear fluorescence). Throughout these studies, the viability of cells was never below 85%, although there was a variable amount of debris particles. In our laboratory, the average cell yield from primary EMT6 tumors is approximately 2 x 10⁶ viable cells/g of tumor tissue (based on over 300 individual tumor cell suspensions). This yield is 2-4 times better than previously reported yields from other laboratories (8-10).

**Phagocytosis.** Phagocytosis of heat-killed Baker’s yeast by adherent cells was measured as previously described (11). Briefly, 50 μl of yeast suspension (1 x 10⁶ particles/ml) and 0.3 ml of guinea pig complement (1:9 dilution in saline) were added to a monolayer of adherent cells on 35-mm dishes in 3 ml of alpha-minimal essential medium supplemented with 10% fetal bovine serum. After 30-min incubation at 37°C, the monolayer was rinsed with warm PBS, counterstained with 0.4% aqueous methylene green for 10 min, air dried, and examined under a light microscope. Two hundred cells were counted under oil immersion optics, and phagocytosis was expressed as the percentage of cells ingesting yeast particles.

**Histological Identification of Granulocytes and Lymphocytes.** Granulocytes and lymphocytes were differentially identified on Wright’s stained cytospin centrifuge preparations. Methanol-fixed slides were stained with a Wright’s stain (Camco Quik Stain; American Scientific Products, Phoenix, AZ) for 2 min, rinsed in distilled water, and air dried. Two hundred cells were counted in random sequential fields under oil immersion light optics.

**Staining of Cellular DNA.** Hoechst 33342, a blue fluorescent dye, was used to qualitatively stain the DNA of viable cells (12, 13). At saturating dye concentrations, cells with tetraploid DNA content exhibit twice the fluorescence of diploid cells (13, 14). Hoechst 33342 dye is a vital stain that enables simultaneous staining with immunofluorescent cell surface marker (13, 14). Hoechst-stained DNA and cell surface immunofluorescent cellular subsets remain viable and can be sorted for functional analyses. Hoechst 33342 staining was performed after detaching cell yield and viability. The cell suspensions were centrifuged at 200 x g for 10 min at 4°C. The supernatant fluid was discarded, and the cell pellet was resuspended to a final concentration of 2 x 10⁷ cells/ml in prewarmed alpha-minimal essential medium supplemented with 10% fetal bovine serum containing 2 mM MOPS (pH 7.0) and Hoechst 33342 dye. Staining was done for 45 min at 37°C in a shaker water bath.

**Monoclonal Antibody Labeling.** Macrophages and granulocytes were identified on the basis of the presence of MAC-1 surface antigen, complement receptors on macrophages and granulocytes (15), and T-lymphocytes were identified on the basis of the presence of Thy-1 antigen (reviewed in Ref. 16). Surface immunoglobulin (on B-lymphocytes or macrophages bearing cytogenic antibodies) was demonstrated by staining with directly fluoresceinized (Fab')₂ fragments of a FGAM heavy and light chain-specific immunoglobulin (TAGO, Inc., Burlingame, CA). It was used at a 1:3 dilution. The presence of MAC-1 surface antigens on macrophages and granulocytes, and of Thy1.2 on T-lymphocytes, was detected by staining with fluoresceinized second reagents. Biotinated Thy1.2 monoclonal antibody (Becton-Dickinson, Mountain View, CA) was used at a 1:10 dilution. The rat anti-mouse MAC-1 monoclonal antibody was produced in culture in our laboratory from hybridoma cells (M1/70.15.11.5; American Type Culture Collection No. T1B128). The appropriate amount (usually 10 μl) of the titered supernatant was used. Fluoresceinized second reagents, FITC:avidin (1:10 dilution) and FGAR, were added to biotinated Thy1.2 and MAC-1 labeled cells, respectively.

**Monoclonal antibody labeling of cells was performed on ice (0°C).** Fifty μl of cells at 2 x 10⁶/ml in alpha-minimal essential medium supplemented with 10% fetal bovine serum containing MOPS and Hoechst 33342 (10 μg/ml) were added to the appropriate antibody. In the case of samples to be stained with biotinated Thy1.2, the cells were first incubated for 10 min with saturating levels (10 μg/ml) of (12) (lgG) mouse myeloma protein (Bionetics Lab Products, Charleston, SC). This was then followed without any washing by the biotinated Thy1.2 reagent. This staining protocol was used to maximize Thy1.2 binding to its specific epitope while substantially reducing nonspecific Fc receptor-mediated binding. After 15 min on ice, the cells were washed by centrifugation (200 x g, 10 min, 4°C). Washes were done using 4 ml HO-PAB. Sodium azide prevents endocytosis and subsequent internalization of fluoresceinated membrane-bound markers. The washed cells were decanted, blotted, and resuspended, and the appropriate second reagent was added. After 15 min on ice, the cells were washed again. Controls included unstained cells and cells stained with second reagents only. After staining was completed, the cells were resuspended in 2 ml of HO-PAB.

Cell suspensions were vortexed briefly and filtered through a 62-μm nylon mesh before flow cytometric analysis. Immediately prior to flow...
analysis, cells were stained for 5 min with propidium iodide (20 μl of 200-
μg/ml stock solution) for exclusion of dead cells (PI labeled) during
analysis.

Flow Cytometry and Cell Sorting. Flow cytometric analysis was
performed on a Los Alamos-designed flow cytometer using a spatially
separated dual laser system (17). Ten thousand events were analyzed.
The instrument is capable of simultaneous measurement of three spatially
separated fluorescence signals in the blue, green, and red wavelength
regions. An argon-ion laser tuned in the UV was used to excite Hoechst
(350–360 nm). PI and FITC were excited at 488 nm with a second argon-
ion laser. The spatial separation of the laser beams permits the sequential
excitation, emission, and collection of transformed fluorescence signals
on appropriately located photomultiplier tubes. The blue fluorescence
emission from Hoechst staining, which is proportional to DNA content,
was measured in the wavelength region of 400–500 nm using a GG400
barrier filter (Schott glass; Melles Griot, Irvine, CA). Green fluorescence
from FITC-stained cells was measured over a 515–545 nm wavelength
region using a 530 band pass filter (Omega Optical, Brattleboro, VT). The red nuclear fluorescence of propidium
iodide-stained cells (dead cells) was measured at >610 nm using an
RG610 long pass filter (Schott glass; Melles Griot, Irvine, CA).

A POP-11/23 computer was used for the data collection and storage
in correlated list mode. Subsets of viable cells stained with specific
monoclonal antibodies were resolved by reprocessing of the multi-
parameter data as previously described (18). Tumor cells were resolved
from host cells based on their DNA content only, since preliminary studies
revealed that EMT6 tumor cells did not bind any of the monoclonal
antibodies used.

In order to morphologically identify the cells bearing receptors for
MAC-1, Thy.1.2, or cells with surface immunoglobulin, they were sorted
into ice-cold 1-ml beakers containing 50 μl of 100% fetal bovine serum.
Coincidence of blue (Hoechst 33342) and green (FITC) fluorescence
signals and anti-coincidence of red (PI) fluorescence were required for
sorting to be initiated. Sort windows were set to selectively sort and
collect viable host cells (PI negative) that displayed green fluorescence
(Chart 1, Row C). The sort rate of cells satisfying these conditions was
500 cells/min. Fifteen thousand to 20,000 cells were collected for each
MAB tested, and cytocentrifuged slide preparations were made. Mac-
rophages, neutrophils, and lymphocytes were identified based on their
differential morphology after staining with Wright’s stain. No attempt was
made to identify cells that could not be unequivocally classified into any
of these three categories.

Statistical Analysis. Statistical significance of results was determined
using a two-tailed t-test for unpaired data with the assumption of unequal
variances in group means (19). All values expressed in the text represent
the mean ± standard error, unless otherwise indicated. Probability values
(P) of <0.05 were considered to indicate significant differences between
data sets. For these studies pooled, tumors from three mice were used
in three separate experiments for each time point.

RESULTS

Resolution of Host and Tumor Cells by Reprocessing of
FCM Data. Row A in Chart 1 shows the FCM data obtained from a
7-day DSC tumor cell suspension labeled with MAC-1 prior to
reprocessing. The debris distribution in Channels 1–28 is exponen-
tially decreasing, so that very little debris contaminates the
DNA distributions. In fact, the last panel shows the distribution
and relative frequency of dead (PI-stained) cells. In Row B, viable
cells were resolved from debris by setting a lower window on
Channel 30 (left histogram) and reprocessing events representing
the viable cells which exclude PI between Channels 0 and 5 in
the right histogram. Eighty-eight % of the cells analyzed were
viable, and 0.6% of the recorded events were found in Channel
29–30. The coefficients of variation of the diploid and tetraploid
DNA peaks after gating out dead cells (PI positive) to eliminate
PI quenching of Hoechst were 17% and 7%, respectively.

The distribution of viable host cells labeled with MAC-1 was
obtained by reprocessing events above Channel 129 in the right
histogram of Row C. In Row D, reprocessing was done on cells not stained with MAC-1 (Channel
0–119).

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(Chart 1A). Both normal diploid host cells and tetraploid tumor cells are resolved. There may be some G2 + M host cells and some aggregates which contaminate the area corresponding to the tetraploid G1 DNA region.

The reprocessing strategy described here was also used to resolve Thy1.2- and FGAM-positive cells (data not shown).

**Total Host Cell Content of Tumors.** The number of host cells infiltrating the tumors was estimated by FCM analysis based on their diploid DNA content. Chart 2A shows the total number of host cells recovered from sponge tumors, blank sponges, and tumors that resulted from DSC injection of tumor cells. At 2 days, after implantation, the number of host cells in blank sponges had stabilized and remained essentially unchanged over the 21-day study period. In contrast, both host cells and tumor cells increased exponentially with tumor age. While sponge tumors elicited an accumulation of host cells that was significantly greater than DSC tumors from Day 7 through Day 21, the rates of increase were parallel. The number of host cells in blank sponges on Day 7 was greater than the number in DSC tumors on Day 7. However, the host cell content of DSC tumors surpassed that of blank sponges by Day 14 and increased progressively up to Day 21.

**Tumor Cell Content of Tumors.** The number of tumor cells within sponge or DSC tumors was resolved by flow cytometric analysis based on their near tetraploid DNA content. Based on the resolution of labeled MAC-1-positive cells described in Chart 1, we estimate about 5% of events in this region may be contaminating host cells. There was no significant difference in tumor growth kinetics between sponge and DSC tumors (Chart 2B). While sponge tumors appeared to contain more tumor cells than DSC tumors, only at Day 7 was the difference significant (P < 0.05). In both cases, tumor cells proliferated rapidly and were in exponential growth through Day 14. A slight reduction in tumor cell growth rate occurred between Day 14 and Day 21.

**Quantitation of Specific Host Cell Types within Tumors and Blank Sponges.** Chart 3 shows the infiltration and identification of host cells into blank sponges and tumors (sponge or DSC) over a 21-day period after implantation of tumor cells. For all of these illustrations, the number of each cell type has been shown rather than the percentage of cells within the tumor on a particular day. In this way, the dynamic changes which occur for each cell population as a function of time can be readily seen. Macrophages identified by their ability to phagocytose yeast constituted the largest proportion of host cells recruited into blank sponges, DSC, or sponge tumors. By Day 2 after implantation, blank sponges had accumulated macrophages, but the extent of macrophage recruitment remained stable with length of sponge residence in the animal thereafter (Chart 3A). In contrast, macrophages in DSC tumors, while lower, increased in parallel to sponge tumors from Days 7 to 21.

Granulocytes constituted the next major host cell type in these tumors. As with macrophages, the number of granulocytes in the blank sponges remained stable from Day 2 through Day 21 (Chart 3B). DSC tumors and sponge tumors showed similar parallel rates of increase from Day 7 to Day 21. While their accumulation profile kinetic values were identical to the other two cell types, lymphocytes made up the smallest number of host cells in blank sponges, DSC tumors, or sponge tumors (Chart 3C). Lymphocyte accumulation in blank sponges peaked at Day 2 and remained at this level on Day 21.

**Monoclonal Antibody Labeling Patterns and Morphology of Sorted Cells.** In order to develop the methodology for flow cytometric identification of host cells and to sort them for future functional analysis, the identification of intratumor host cells was

![Graph of Total Host and Tumor Cells](image)

**Chart 2.** Cellular composition of EMT6 tumor. Sponge tumor (Δ), blank sponge (□), and DSC tumor (○). Sponges were implanted 2 days prior to injection of tumor cells to provide equilibrium of the microenvironment; Days 0, 7, 14, and 21 on the x-axis represent 2, 9, 16, and 23 days, respectively, postimplantation of sponge. Approximately 1 × 10^6 tumor cells were injected into mice directly under the skin (DSC) or into a preimplanted gelatin sponge. At 7, 14, and 21 days postinoculation, tumors were excised and disaggregated. The actual number of cells recovered from the tumor or sponge was determined by multiplying the total number of cells by the fraction of viable host or tumor cells in the sample. Points, mean of at least 3 experiments, each involving 3 pooled tumors or blank sponges for each time point; bars, SE.

**Table 1**

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<th>Lymphocyte</th>
<th>Neutrophils</th>
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<td>1.0</td>
<td>0.0</td>
<td>5.5</td>
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<tr>
<td>Thy1.2</td>
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...also accomplished by staining cell suspensions with MAbs specific for MAC-1 (for macrophages and granulocytes) surface immunoglobulin (for B-cells) and Thy1.2 antigen (for T-cells). The results are shown in Chart 4 and Table 1. The kinetics of cell accumulation within the three experimental groups is like that found for the morphological identification presented in Chart 3. Thus, blank sponges had a stable cellularity throughout the 21-day observation period, while the accumulation of cells within the DSC tumors on sponge tumors paralleled each other. The number of MAC-1-positive cells correlated with the number possessing phagocytic activity except for 21-day sponge tumors, where there were more macrophages positive for MAC-1 than were phagocytic. This may be due to debris-laden macrophages in the fully consolidated sponge tumors, which still expressed the MAC-1 epitope but may have lost phagocytic activity. The numbers of cells positive for the slg marker (FGAM) or Thy1.2 increased similarly with tumor age in both DSC and sponge tumors (Chart 4, B and C).

Following staining of the cell suspensions with cell-specific MAbs, positively labeled cells were sorted for morphological identification. The results from a typical sort are shown in Table 1. Most of the cells bearing the MAC-1 epitope were macrophages (95.5%). A considerable proportion (93.5%) of sorted slg-positive cells were also macrophages; virtually no slg-positive cells were lymphocytes indicating a lack of B-cells. The Thy1.2-positive cells also consisted primarily of macrophages as only 22% were lymphocytes.

DISCUSSION

This study has demonstrated the use of a gelatin sponge model for studying tumor growth. Blank sponges lacking tumor cells induced an inflammatory response in the animals harboring them. This influx occurred prior to Day 2 (our first observation point), and the cell content and phenotype remained stable thereafter. Superimposed on this background is the response to the tumor. In the presence of proliferating tumor cells, the host response was augmented, and there was a continuous influx of macrophages, granulocytes, and lymphocytes into the tumor site over and above that induced by the sponge itself. This finding is in agreement with the hypothesis that a host recognizable event(s) occurs in response to the presence of tumor cells that is distinct from the foreign body reaction elicited by the blank sponge. The data indicate that the kinetics of both tumor cell and host cell increase in sponge and DSC tumors are identical. Of the host cell types infiltrating the tumors, macrophages constituted the largest fraction, followed by granulocytes and lymphocytes. The sponge model is a recoverable arena that enables one to study host-tumor cell interactions during the early stages of tumor development or during immunological or therapy-induced rejection when the tumor is not palpable. Events which occur during these times have not been studied. At these stages, infiltrating putative effectors attracted to the tumor site are trapped within the sponge matrix and can be recovered for morphological and functional analysis. As a progression of our work using the sponge model, for example, we have found in animals expressing concomitant tumor immunity that cytotoxic T-lymphocytes are selectively recruited to preimplanted sponges challenged with tumor cells. The interactions would not be amenable to study in DSC tumors because the total cell burden at the tumor site would not be large enough to manifest itself as a palpable tumor that could be recovered for analysis.

Several important heretofore undescribed observations have been made in this study. The surface immunoglobulin-bearing cells (slg positive) detected by flow cytometric analysis and sorting were predominantly macrophages and not B-cells. Most of the Thy1.2-positive cells were also macrophages (68%). The remaining cells were lymphocytes (22%) or cells of unidentified morphology (10%). While it is well known that the Thy1.2 epitope is present on T-lymphocytes, Basch and Berman (20) recently demonstrated the presence of the Thy1.2 epitope on bone marrow-derived immature monocytes and granulocytes. Our studies confirm their finding and suggest that a subpopulation of tumor-associated macrophages also bears the Thy1.2 epitope. The functional relevance of the Thy1.2-positive and slg-positive subsets of macrophages requires definition.

The progressive recruitment of host macrophages and lymphocytes that accompanies tumor progression is well documented. In an earlier study (21), we showed that the macrophage content of mice bearing the EMT6 mammary carcinoma increased with tumor age. Evans and Eidlen (22) demonstrated that certain spontaneous or chemically induced murine sarcomas and carcinomas manifested an exponential increase in tumor-infiltrating macrophages until a critical tumor size was attained, after which the rate of influx was diminished. A similar response to increasing tumor size has been described for infiltrating lymphocytes in primary Moloney sarcoma virus-induced tumors in mice (23).

We believe that the greater extent of host cell infiltration into sponge tumors is due, in part, to vascularization that had occurred in the preimplanted sponges prior to the time of tumor cell inoculation (1). Thus, there were already some host cells present when the tumor cells were injected. The retention of tumor cells at the inoculation site by the sponge matrix stabilizes the initial tumor burden to which the host-immune repertoire is directed. Conceivably, the maintenance of the tumor burden may determine the extent of host cell recruitment into the tumor (22).

While it is unequivocal that an increase in the intratumor...
content of macrophages and lymphocytes occurs during tumor progression, the mechanism(s) responsible for their accumulation is still speculative. Intratumor macrophages, on which the most data are available, are generally assumed to derive from bloodborne monocytes either by passive transport via the circulatory system (24) or in response to chemotactic factors present at the tumor site (reviewed in Ref. 25). Proliferation of intratumor macrophages (23, 26, 27) and perhaps lymphocytes (28) contributes to the overall mononuclear cell content of a consolidating tumor. Chemotactic factors elucidated by neoplastic cells have been indirectly linked with macrophage accumulation at a tumor site. Bottazzi et al. (29) recently showed that human or mouse tumors produced factors in their supernatant fluids that were chemotactic in vitro for human monocytes and mouse macrophages, respectively. They also reported a correlation between chemotactic activity of certain tumors and the extent of macrophage infiltration in vivo.

The reports of Bottazzi et al. (29) and others (30) that have attempted to elucidate the mechanism(s) of leukocyte accumulation within a tumor suffer from extrapolation of in vitro observations to explain in vivo phenomena. As a matter of fact, evidence of function in vivo of these factors still remains to be provided.

The sponge system described here would provide a defined arena for the injection of factors, such as chemotactic factors, angiogenesis factors, host defense cell-activating factors, or tumor cell fragments, into the test animal to assess their role in tumor progression or rejection. Furthermore, the sponge can also provide a source of these factors. Since the sponge can be retrieved at any time, it offers a valuable means to study host-tumor cell interactions at the early stages of tumor progression or during the rejection process when the tumor is neither visually discernible nor palpable.

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