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Morphological and Functional Characteristics of Cells Infiltrating and Destroying Tumor Multicellular Spheroids in vivo* **

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Summary. EMT6 mammary sarcoma cells were grown in vitro as multicellular spheroids to model for the heterogeneity of microenvironments and structural changes which develop in many tumors, including micrometastases. Spheroids of 700–900 μm diameter were implanted into and recovered at different times from the peritoneal cavities of sensitized or nonsensitized allogeneic and syngeneic mice. The colony forming efficiency of spheroid tumor cells recovered at 24 and 48 h from sensitized allogeneic mice was markedly decreased as compared with those from nonsensitized allogeneic or syngeneic animals. These recovered spheroids were extensively infiltrated by both lymphocytes and macrophages, which ultrastructurally had very close membrane associations with tumor cells. Host cells recovered from spheroids exhibited cytotoxic activity in an in vitro ^{51}Cr release assay. Thus, multicellular spheroids in vivo provide a unique experimental model to study the functional capacity of host cells within a spheroical tumor. Although lacking the stroma and the vasculature of in vivo solid tumors, this model does have many similarities to in vivo tumors and is thus suitable for studying the tumor cell-host cell interactions within the tumor microenvironment. In addition, the system offers the potential for quantitative study of the effects of treatment modalities on tumor cell-host cell interactions.

Key words: Immunology – Ultrastructure – Multicellular spheroids – Tumors.

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Introduction

A better understanding of the complex interactions between tumor cells and cells of the host immune system is essential for the rational application of specific immunotherapy and combined therapy with known immunosuppressive agents, such as radiation and drugs. This understanding is confounded not only by the complexities of the interrelationships of the different arms of the immune system, but also by probable heterogeneities in both cellular structure and microenvironments in solid tumors even of micrometastatic size.

The complex interactions between tumor cells and the cells of the immune system often make interpretations of in vivo experiments extremely difficult. Although studies with in vitro systems allow for analysis of individual components, it is critical that in vitro systems accurately reflect in vivo phenomena.

Immunological response to allografts and to at least some tumors involves the generation of specific cytotoxic effector cells (Cerottini et al., 1974; Herberman, 1974). In vitro assays have been extensively used to detect and quantitate the activity of these cells. Although informative, these two-dimensional cell mediated cytotoxicity assays assess only the ability of such cells to recognize and attack a homogeneous, exponentially growing population of tumor cells under optimal culture conditions. Such assays do not address a number of important questions including whether or not a) the effector cells are capable of infiltrating a solid tumor, b) their function is affected by the changes in the microenvironmental oxygen tension often found in rapidly growing tumors (Baserga, 1971; Fowler, 1972; Kaplan, 1974; Gillespie et al., 1977), and c) the heterogeneous mixture of tumor cells in various stages of growth and cell cycle will all be equally recognized and susceptible to lysis.

An approach to these questions which has been used recently by several groups is to disaggregate solid tumors (Gillespie et al., 1977; Haskill et al., 1975; Pross et al., 1976; Russell et al., 1976) or organ allografts (Strom et al., 1977; Tilney et al., 1976) and attempt to identify morphologically and functionally the infiltrating lymphoid cells. However, disaggregation of solid tumors is complex and generally requires both mechanical and enzymatic treatment, often with low viable cell recovery. An approach to this problem recently developed in our laboratory has been the study of the interactions of lymphoid host cells with multicellular spheroids, an extensively studied in vitro tumor model system (Sutherland et al., 1976).

Spheroids grown under controlled conditions develop a three-dimensional structure and alterations in growth kinetics and cellular microenvironments similar to many tumors in vivo, especially micrometastases. Although tumor cell population heterogeneity produced by structural and microenvironmental factors is known to play a major role in determining the properties and responses of cells to radiation and chemotherapy (Sutherland et al., 1976), the extent to which such factors may also contribute to modifications of responses to the immune system is unknown. Since spheroids can be readily dissociated to obtain viable single cell suspensions, a system involving intraperitoneal spheroid implantation allows for analysis and quantitation of both the infiltrative and functional capacities of various types of immune cells.

The susceptibility of mammary tumor spheroid cells to infiltration and lysis in vitro by cytotoxic T lymphocytes sensitized to alloantigens in a mixed lymphocyte culture has been reported (Sutherland et al., 1977). The kinetics of tumor cell killing in spheroids was much slower than in standard single cell cytotoxicity assays, and the spheroids contained a significant fraction of resistant cells. Lymphocytes prelabeled with tritiated thymidine were observed by autoradiography to have infiltrated the spheroids. In this report, we have extended these studies to assess whether or not resistant spheroid cells are also present in vivo and the extent to which host lymphoid cells infiltrate.

Spheroids of EMT6 mammary sarcoma cells grown in vitro for 2–3 weeks were implanted into and recovered from the peritoneal cavities of nonsensitized and sensitized (alloimmune) mice. The kinetics of host immune cell infiltration and the extent of in situ destruction of the tumor cells in response to strong allogeneic antigens provide information on the types of infiltrating cells and their ability to function in vivo in the microenvironment of tumor-like structures. They also provide a basis for future studies of syngeneic tumor-specific antigens and the influences of drugs and radiation on these responses.

The results presented here demonstrate that spheroids provide a useful system for assessing immunological responses in vivo in tumor-like microenvironments and are in agreement with the recent observations of MacDonald et al. (1978a, b) on the kinetics of host cell infiltration and destruction of tumor cells. We have extended these observations to identify morphologically two types of host cells involved in tumor cytotoxicity (or tumor cell destruction).

Materials and Methods

Mice

C57BL/6, C3H/HeJ and DBA/2 adult mice were obtained from the Jackson Breeding Laboratories, Bar Harbor, Maine. BALB/cKa mice were obtained from Bio Breeding Labs of Canada, Ltd., Ottawa, Ontario, Canada.

Growth of Tumor Cells and Spheroids

P815 mastocytoma cells (DBA/2 origin, $H\text{-}2^d$) were maintained by twice weekly passage in vitro. Growth of spheroids of EMT6 mammary sarcoma cells (BALB/c origin, $H\text{-}2^d$) was initiated by placing 2.5×10^4 exponentially growing cells into 60 mm non-tissue culture petri dishes (Labteks). This nonadherent surface favors intercellular contact and results in small spheroids of 100–150 μm diameter after 3–4 days. These were then transferred to 300 ml spinner flasks(100 rpm) with 125 ml of Eagle's basal medium (BME) supplemented with 15% fetal bovine serum (FBS), and antibiotics penicillin (100 units/ml) and streptomycin (100 μg/ml). The medium and gas phase were replenished daily. After 4 more days of growth when the spheroids had reached a diameter of 300–400 μm, they were sorted to obtain a homogeneous sized population, and 500 spheroids were placed in spinner flasks (190 rpm) with 200 ml of medium. Spheroids of 700–900 μm diameter obtained after 2–3 weeks growth were used in these experiments.

Immunizations

Mice were immunized by a single intraperitoneal injection of 1×10^7 viable P815 mastocytoma cells 15 days prior to use.

Spheroid Implantation and Recovery

Spheroids (40–60 per mouse) were implanted into the peritoneal cavity using an 18 gauge needle. After 24 or 48 h, the mice were sacrificed by cervical dislocation and the spheroids were recovered by repeatedly flushing the peritoneal cavity with Hanks balanced salt solution (BSS) containing 10 units/ml heparin. A lower number of spheroids was generally recovered at 48 h from the alloimmune mice (20–60%) than from the normal mice (40–85%). In the normal mice, the spheroids retained their original size and spheroidal shape; however, in the alloimmune mice, the spheroids were often small and misshapen into elongated elipsoid structures. The spheroids were separated from the peritoneal cells (PC) by gravity sedimentation. After three washings with BSS, the spheroids were dissociated into a single cell suspension by trypsinization (0.25% trypsin for 12 min at 37° C). Cell counts of the trypsinized spheroid associated cells (SAC) were obtained using an electronic particle counter set for optimal counting of particles the size of EMT6 cells and by parallel hemocytometer counts in which both tumor cells and lymphoid cells were distinguished on the basis of size and internal structure. The hemocytometer count of tumor cells was always in close agreement with the cell count obtained using the electronic particle counter. The peritoneal cells, which contained very few tumor cells, were washed by centrifugation and counted in a similar manner.

Morphology

The EMT6 spheroids were harvested from the peritoneum either 24 or 48 h following implantation, fixed for 2 h in 2% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2), and subsequently processed as previously described (Penney at al., 1977). For comparison, control spheroids grown solely in vitro were harvested at corresponding time periods. Thick sections (0.5–1.0 μm) were stained with methylene blue and azure II for light microscopy. Adjacent thin sections (40–80 nm) were stained with lead citrate and uranyl acetate for observation using a Zeiss 10A electron microscope.

Colony-Forming Efficiency (CFE) Assay

The CFE of the SAC was determined by plating the cells at four dilutions in 60 mm petri dishes (5 plates/dilution) in 5 ml of BME supplemented with 15% FBS. After 10 days incubation, the plates were washed, stained with methylene blue, and scored for colonies.

Cytotoxicity Assay

The cytotoxic potentials of the PC and SAC were determined using a ^{51}Cr release assay. Since P815 and EMT6 share *H-2*^{d} alloantigens, P815 was used as the target cell because of its greater susceptibility to lysis (Sutherland et al., 1976) and the extensive characterization of this cell line as a target (Cerottini et al., 1974). Exponentially growing P815 cells (5×10^6), were harvested from culture, washed, and resuspended in 1 ml of RPMI 1640 medium supplemented with 5% FBS and 10 mM (HEPES (N-2-hydroxyethylpipetazine-N-2-ethane sulfanic acid) buffer (assay medium). We added 200 μCi $Na_2\ ^{51}CrO_4$ (Amersham/Searle Corporation, Arlington Heights, ILL) in 0.2 ml of saline solution, and the cells were incubated for 30 min at 37° C. After three washings, the target cells were resuspended in assay medium, counted, and adjusted to 4×10^5 cells/ml. For the ^{51}Cr release assay, varying numbers of PC or SAC were mixed with 1×10^4 target cells in 0.2 ml of assay medium in triplicate wells of Linbro JS-FB 96-TC microtissue culture plates. Following incubation of the plates at 37° C for 3 h, 0.1 ml of supernatant was removed from each well and radioactivity was counted using a well-type gamma counter. Spontaneous release controls contained target cells, but no lymphoid cells. Spontaneous release was generally less than 8.0%. Maximum release was determined by incubating target cells in 1.0 N HCl. Percent specific lysis was calculated using the following formula:

$$\frac{\text{experimental } ^{51}Cr \text{ release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100.$$

Cytocentrifuge Preparations

For morphological analysis, 0.5 ml of a single cell suspension containing 5×10^5 cells/ml of the dissociated spheroids recovered after 24 or 48 h in vivo was centrifuged for 5 min at 500 rpm on a Shandon-Elliott cytocentrifuge, airdried and stained with Wright's stain.

Results

The usefulness of the multicellular spheroid for in vivo studies of host cell infiltration and function within the microenvironment of a tumor was assessed by implanting spheroids into the peritoneal cavities of mice and recovering them at various times post-implantation. CFE assays were used to assess the viability of the tumor cells from the recovered spheroids. Light and electron microscopic studies and ^{51}Cr release cytotoxicity assays defined the nature of the host cell infiltration and confirmed the functional capability of infiltrating cells.

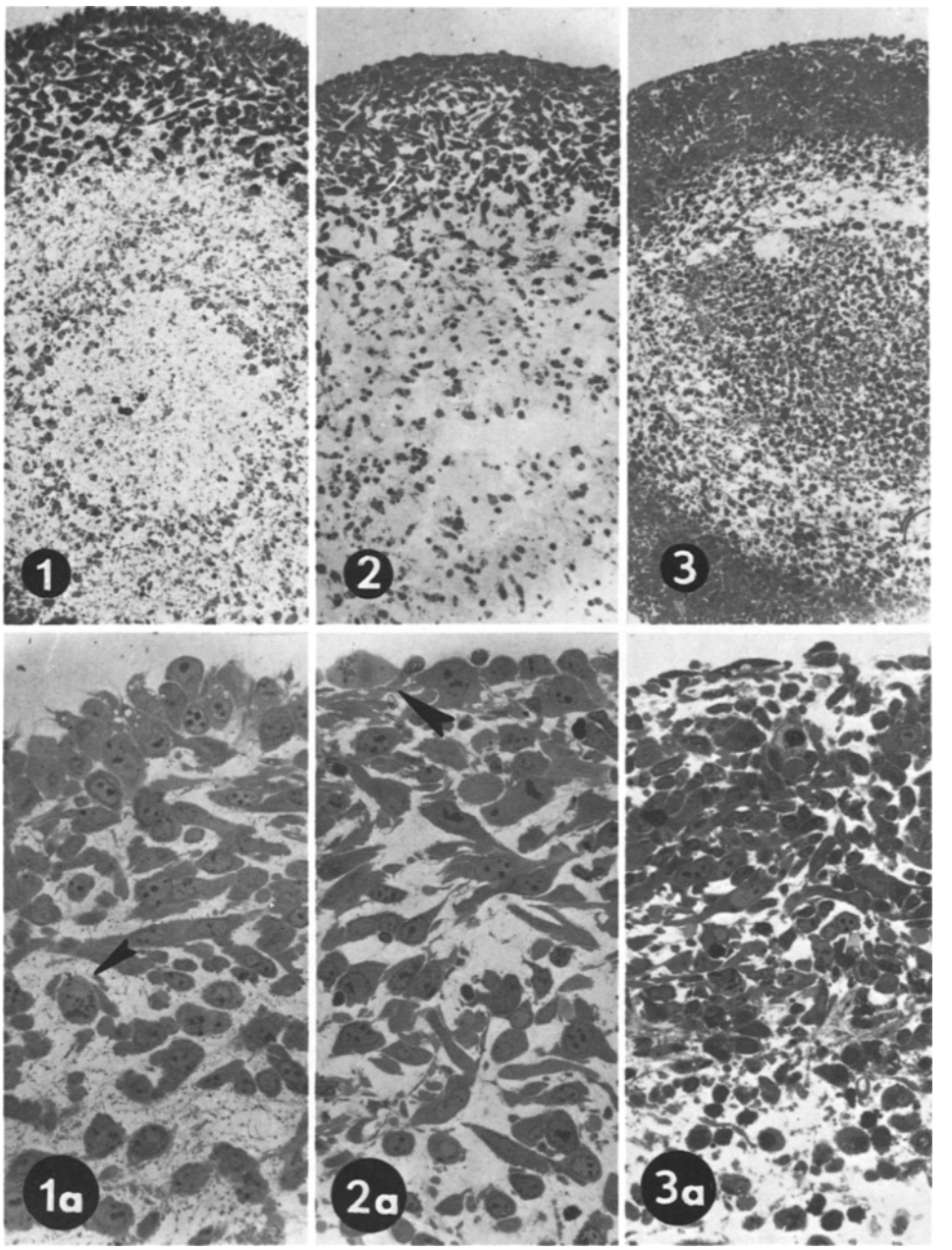
Histology

Multicell spheroids of EMT6 cells develop necrotic centers at sizes greater than about 500 μm diameter, due primarily to the limited diffusion of oxygen and nutrients (Sutherland et al., 1976). Thus, spheroids of the sizes used in this study contained rather extensive areas of central necrosis (Fig. 1). The outer viable region of spheroids grown continuously in suspension in vitro contained viable cells with numerous mitotic figures. The tumor cells were variable in size and shape, reflecting different states of growth and/or metabolic activity.

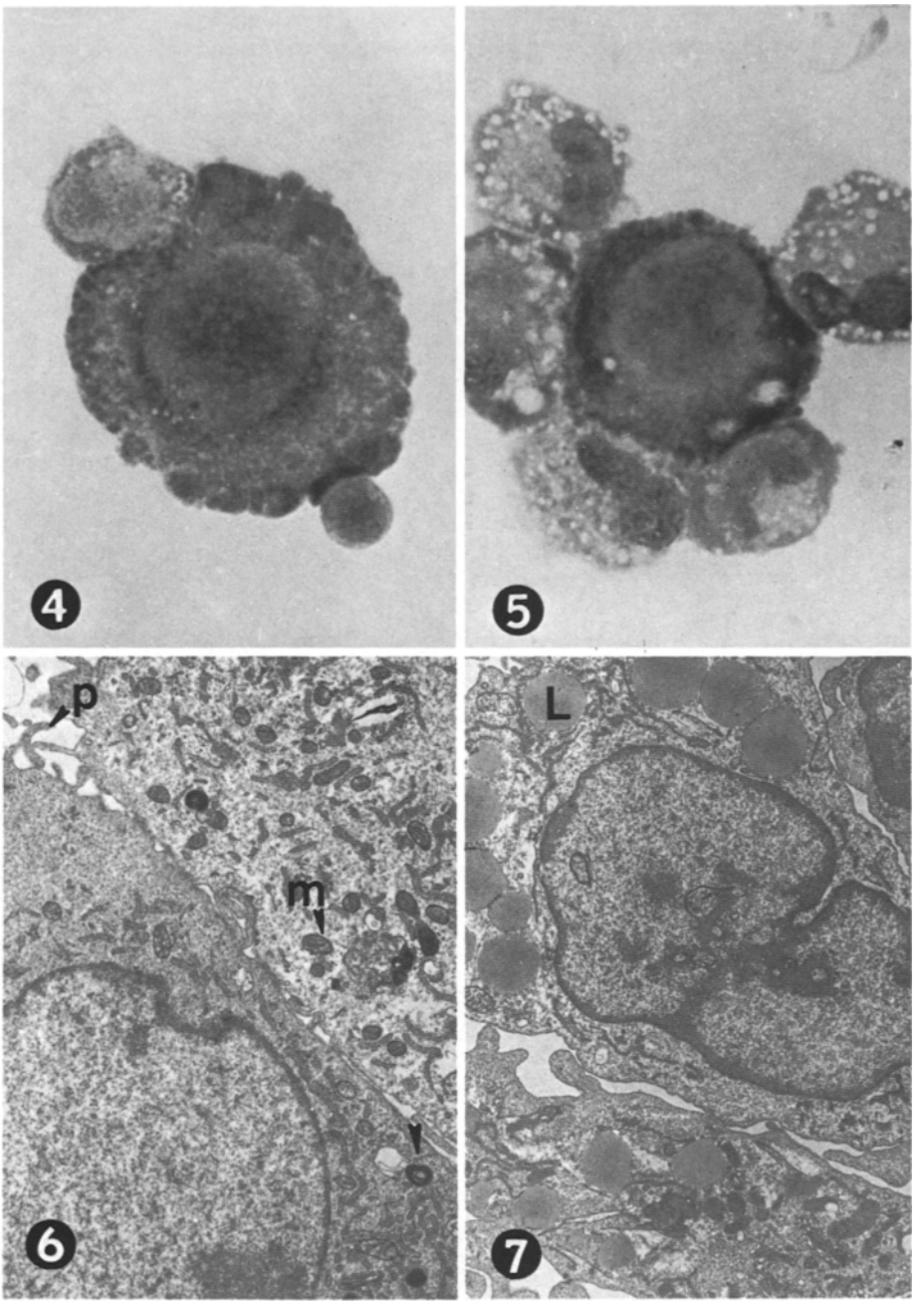
Spheroids recovered from the nonsensitized allogeneic mice were similar histologically to spheroids grown in vitro, except for the infiltration of small round cells (lymphocytes) and histiocytes (macrophages) into the central necrotic zone and the occasional presence of lymphocytes in the outer growing zone of viable tumor cells (Fig. 2). Numerous mitotic tumor cells were present. Lymphocytes and macrophages were randomly distributed and lacked close associations with tumor cells.

Spheroids recovered from sensitized allogeneic mice exhibited pronounced infiltration of both the outer zone and the central necrotic area by lymphocytes and macrophages, with increases in amorphous necrotic material and degenerating and necrotic tumor cells (Fig. 3). Close juxtaposition of lymphocytes and macrophages to tumor cells, often with multiple host lymphoid cells surrounding one cell, was common. In contrast to spheroids from nonsensitized animals, tumor cells in mitosis were extremely rare, and free nuclei from lysed tumor cells were common.

The association between the EMT6 tumor cells and the infiltrating host cells was further investigated using cytocentrifuge preparations of the dissociated spheroids recovered from sensitized mice. Macrophages and lymphocytes were the major infiltrating cell types identified, while polymorphonuclear leukocytes were present in much lower numbers. Macrophages or lymphocytes were often



Figs. 1–3 and 1a–3a. Light micrographs of spheroids grown in vitro **(Fig. 1 and 1a)**, or recovered from the peritoneal cavities of nonsensitized **(Fig. 2 and 2a)** or sensitized **(Fig. 3 and 3a)** mice 24 h following implantation. Arrows in **Fig. 1a and 2a** indicate cells in mitosis. The upper micrographs **(1–3)** are $\times 400$, and the lower **(1a–3a)** are $\times 1{,}000$



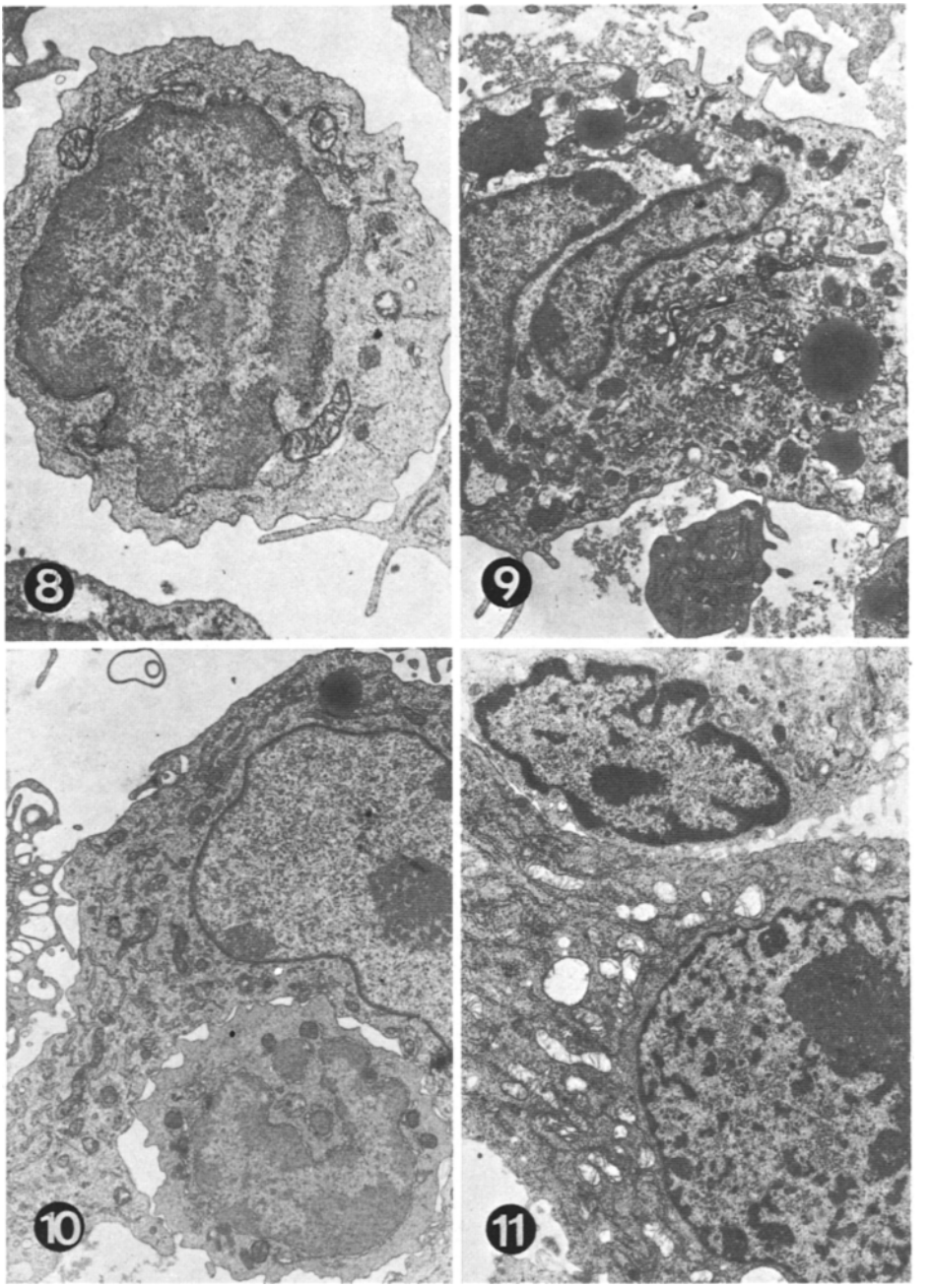
Figs. 4 and 5. Cytocentrifuge preparations of dissociated spheroids recovered after 48 h from the peritoneal cavity of sensitized mice which had received an intraperitoneal injection of carbon particles 48 h prior to spheroid implantation

Fig. 4. A macrophage and lymphocyte in close membrane contact with an EMT6 tumor cell. ×3,000

Fig. 5. An EMT6 tumor cell surrounded by host macrophages in a "rosette" configuration. ×3,000

Fig. 6. Cells from a spheroid grown in vitro for 24 h postimplantation time. Numerous microvillous projections *(p)* characterize the cell surface. Variability in cytoplasmic density exists with cells of lesser density having fewer ribosomes. Dense bodies (arrowhead), some of which are lamellar, and irregularly arranged profiles of the smooth or agranular endoplasmic reticulum are present. The mitochondria (*M*) of the tumor cells usually possess a sparse cristaform internal structure. ×6,325

Fig. 7. Cells from EMT6 spheroid implanted into the peritoneal cavity of sensitized mice for 48 h exhibit marked storage of cytoplasmic lipid-like material *(L)*, which appears to be prescursor of lamellar structures. ×6,325



Figs. 8–11. EMT6 spheroids implanted into the peritoneal cavities of sensitized mice and recovered 48 h later. Lymphocytes and histiocytes are observed within the spheroids, both free (Figs. 8 and 9, respectively). Note the areas of contact between the tumor cell and the lymphocyte and histiocyte, respectively, **Fig. 8:** ×4.785; **Fig. 9:** ×3,885; **Figs. 10 and 11,** ×6,325

Fig. 8. A lymphocyte containing cristaform mitochondria and occasional profiles of the endoplasmic reticulum. ×4,785

Fig. 9. Portion of histiocyte within the spheroid. Note the lipid-like structures, prominent Golgi complex, intact cristaform mitochondria and pleomorphic dense bodies. ×3,885

Fig. 10. A lymphocyte is present within a tumor cell indentation or invagination. Note the multiple lymphocyte microvilli and points of lymphocyte-tumor cell contact. ×6,325

Fig. 11. A histiocyte *(above)* is juxtaposed to a tumor cell *(below)* within the implanted spheroid. Extensive membrane apposition characterize the contact. ×6,325

found in close membrane contact with tumor cells (Fig. 4). Several macrophages (Fig. 5) were often found surrounding a single tumor cell in a "rosette" configuration.

Electron Microscopy

Tumor cells in the viable zones of EMT6 spheroids grown in vitro or recovered 24 h following implantation into nonsensitized mice were remarkably similar (Fig. 6). They possessed large pleomorphic euchromatic nuclei with prominent nucleoli. Their cytoplasm contained irregularly-arranged profiles of granular endoplasmic reticulum, mitochondria with poorly developed membranous interna, numerous free ribosomes, sparse Golgi material and occasional lysosomal dense bodies. Cell membranes possessed extensive microvillous projections which intertwined with those of adjacent cells and formed numerous intercellular contacts. Tumor cell cytoplasm varied in staining density with both ribosome rich and poor cells.

Tumor cells in spheroids recovered within 24 or 48 h (Fig. 7) following implantation into sensitized mice differed from those from nonsensitized animals or in vitro controls. Intercellular spaces between tumor cells were increased, lessening their appositional contact. Microvillous projections remained prominent, but were not as closely intermingled with those of lymphocytes and histiocytes. Tumor cell nuclei contained occasional inclusions (Fig. 7), and their cytoplasm contained increased lipid-like material in droplet form. Lymphocytes with ribosome rich, membrane poor, lysosome free cytoplasma (Fig. 8) were frequently found in close apposition to tumor cells, often within cytoplasmic invaginations and in close membrane contact (Fig. 10). Histiocytes, characterized by prominent Golgi zones, cristaform mitochondria, numerous pleomorphic lysosomal dense bodies, granular endoplasmic reticulum and numerous small vesicles (Fig. 9) also closely abutted tumor cells (Fig. 11). However, most of the tumor cells did not appear to undergo cytoplasmic degeneration prior to cell death.

Colony-Forming Efficiency (CFE)

The effect of implantation in vivo on the viability of the spheroid tumor cells was investigated by determining the CFE of the cell suspensions obtained by dissociating the recovered spheroids. Cell numbers for those assays were based on counts obtained from the electronic particle counter. Spheroids recovered from nonsensitized syngeneic (BALB/c) or allogeneic (C57BL/6) mice after 24 or 48 h contained tumor cells whose CFE did not differ from control spheroids held in vitro for comparable times (Table 1). However, because the environment within the peritoneal cavity is markedly different from in vitro culture conditions, fewer EMT6 cells were recovered from the in vivo implanted spheroids, so that if the number of colonies per spheroid was calculated the values were much lower for the in vivo spheroids. In contrast, cell suspensions of spheroids

Table 1. Effect on EMT6 spheroids of implantation in the peritoneal cavity of syngeneic or allogeneic mice[a]

Mice	Colony-forming efficiency (% ± S.D.)		Colonies per spheroid ($\times 10^3$)	
	24 h	48 h	24 h	48 h
In vitro control[b]	41.4 ± 1.5	37.8 ± 5.3	33.1	35.2
Nonsensitized BALB/c	37.9 ± 6.4	33.2 ± 0.6	13.5	20.3
Nonsensitized C57BL/6	31.0 ± 3.5	31.6 ± 0.5	14.7	15.3
Sensitized C57BL/6	9.2 ± 3.5	3.1 ± 0.6	3.9	0.48

[a] EMT6 spheroids (0.833 mm, dia. ± 0.077 S.D.) were implanted into groups of 3 mice and recovered 24 or 48 h later, pooled, trypsinized and assayed for surviving clonogenic tumor cells
[b] Control spheroids which were not implanted in mice were left growing in the spinner flasks and samples were trypsinized and assayed at 24 and 48 h

Table 2. Effect of priming with different *H-2*^{d} tumors on the in vivo destruction of EMT6 spheroids[a]

Mice	Colony-forming efficiency (% + S.D.)	Colonies per spheroid ($+10^3$)
In vitro control[b]	45.7 ± 3.7	23.3
P815 sensitized C57BL/6	3.5 ± 0.4	1.1
EMT6 sensitized C57BL/6	5.0 ± 0.3	1.7

[a] EMT6 spheroids (0.815 mm, dia. ± 0.106 S.D.) were implanted into the peritoneal cavities of groups of 3 mice, recovered 48 h later and then pooled, trypsinized and assayed for surviving clonigenic cells
[b] Control spheroids which were not implanted in mice were left growing in the spinner flasks and samples were trypsinized and assayed at 48 h

recovered from sensitized mice after 24 h had a markedly lower CFE than those of the normal controls and an even greater reduction was seen after 48 h. The similar reduction in the number of colonies per recovered spheroid indicates that the lower CFE was due to an actual loss of viable tumor cells, not merely to their dilution by infiltrating cells. Furthermore, this decrease in CFE does not occur in the plates during the incubation period for colony formation due to admixed lymphoid cells since removal of the majority of cytolytic cells by treatment with ATS and complement does not alter the CFE. Also, no difference was observed in CFE when recovered SAC were plated in methyl cellulose in order to separate lymphoid cells physically from tumor cells (Sutherland et al., 1977). Destruction of the EMT6 spheroids were due to cells recognizing the *H-2*^{d} alloantigens rather than any tumor specific antigens in that the CFE of the EMT6 spheroid cells was reduced to the same extent in animals primed to the P815 cells as to the EMT6 cells (Table 2). However, this destruction is immunologically specific for the *H-2*^{d} alloantigen in that when mice are immunized with EL4 tumor cells, syngeneic to C57BL/6 (*H-2*^{b}), there is only a slight decrease in plating efficiency of the recovered spheroids while there is marked decrease in those recovered from the specifically

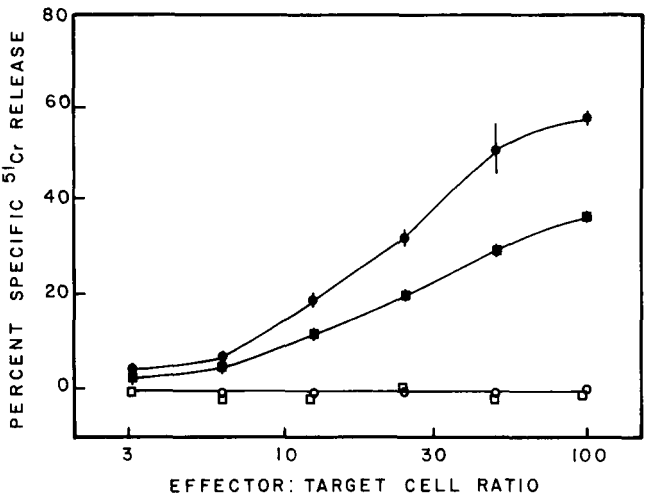


Fig. 12. Presence of cytotoxic cells within spheroids. EMT6 spheroids were recovered 24 h after implantation in nonsensitized or sensitized C57BL/6 mice. The trypsinized spheroid associated cells *(SAC)* and the peritoneal cells *(PC)* were assayed for cytotoxicity on ^{51}Cr labeled P815 cells at several effector to target cell ratios. ●—●, sensitized PC; ■—■, sensitized SAC; ○—○, nonsensitized, PC; □—□, nonsensitized SAC

Table 3. Immunological specificity of spheroid destruction[a]

Mice	Colony-forming efficiency	Colonies per spheroid ($\times 10^3$)
Nonsensitized C3H/HeJ	28.2±5.5	24.8
EL4 sensitized C3H/HeJ	24.0±3.1	18.6
P815 sensitized C3H/HeJ	4.2±0.2	2.3

[a] EMT6 spheroids (0.941 mm dia. ±0.076 S.D.) were implanted into the peritoneal cavities of groups of 3 mice, recovered 48 h later and then pooled, trypsinized and assayed for surviving clonigenic cells

(*H-2*^{d}) alloimmune mice (Table 3). This difference was not due to a failure of the immunization protocol in that lymphoid cells from the peritoneal cavity of both types of alloimmune mice lysed the appropriate target cell in a ^{51}Cr release assay performed at the time of spheroid recovery.

Cytotoxic Potential of PC and SAC

The cytotoxic potential of the host cells infiltrating the spheroids as well as those remaining free in the peritoneal cavity was assessed by using the SAC or PC as effector cells in a 3 h ^{51}Cr release assay for cytotoxic cells using DBA/2 P815 cells (which share the same major histocompatibility antigens of EMT6 cells) as target cells. Cell numbers used in these experiments were based on total cell counts (tumor cells and infiltrating host cells) obtained

from the hemocytometer. Cytotoxic activity was present in both the SAC and PC of the alloimmune mice after 24 h while the nonimmune controls were inactive (Fig. 12). When the SAC and PC were recovered 48 h after spheroid implantation, the level of cytotoxicity in each population was similar to that present at 24 h (data not shown). The data in Fig. 12 are characteristic of 12 similar experiments in which the level of cytotoxicity present in the SAC was generally lower than that in the PC population. However, it is important to consider that the cell numbers used in these assays were based on total cell counts (tumor and infiltrating cells). The PC contain only a minimal number of tumor cells ($< 0.003\%$ CFE based on plating 1×10^6 cells), while the SAC contain many more EMT6 cells. Thus, the lower activity of the SAC observed in the ^{51}Cr release assay is due not only to the presence of tumor cells but may also be related to tumor cells acting as unlabeled inhibitors in competing with the labeled P815 target cells for lysis by the cytotoxic lymphoid cells.

Discussion

As the spheroid grows in overall diameter in vitro, there are varying states of cellular activity, as exemplified by the outer surface cells which contain more numerous microvillous projections, a subjacent mitotic zone, and an inner hypoxic zone adjacent to the zone of necrosis. Spheroids recovered following in vivo implantation exhibited similar changes. It is considered that the variable sizes, shapes and densities of the cytoplasm of the tumor cells may reflect these functional states.

The spheroids implanted to the peritoneal cavities of sensitized mice exhibited a greater infiltration of host cells in the inner zones of the spheroid. The morphological differences, when compared to spheroids recovered from in vitro experiments or from non-sensitized mice, were predominantly limited to intercellular and plasmalemmal alterations. Host cells, especially lymphocytes and histiocytes, were commonly juxtaposed to tumor cells wherein numerous points or areas at contact between tumor and host cells were observed. Attempts are underway to determine whether or not these contacts are indeed communicating junctions. Nevertheless, where host cell infiltration was increased, host cell-tumor cell contacts were common, suggesting these host cells play a major role in either inducing or assisting tumor cell destruction. Interestingly, most tumor cells did not appear to undergo any marked cytoplasmic degeneration indicative of cell death following the establishment of contact by host cells. This phenomenon appears to lend credence to the view that tumor cytotoxicity during host cell attack involves membrane breakdown or dissolution, permitting the observed rapid tumor cell cytoplasmic extrusion (Sanderson, 1976).

Although these experiments demonstrate that spheroids implanted in sensitized allogeneic mice do contain lymphocytes and macrophages and that some component of these infiltrating cells is capable of lysing allogeneic tumor cells, the exact nature of the effector cell(s) and the relative contribution of lymphocytes and macrophages to tumor cell cytotoxicity were not defined by these studies. Previous in vitro studies (Sutherland et al., 1977) showed that cytotoxic

T lymphocytes generated from spleen cells in a mixed lymphocyte culture were capable of infiltrating spheroids and destroying the tumor cells. However, about 20% of the tumor cells were resistant. In contrast, the in vivo experiments reported here demonstrated no resistant cells. This difference could be due to the presence of host infiltrating cells, other than T lymphocytes serving as effector or helper cells. The additional killing in vivo could, however, also be due to other factors, such as differences in the activity of the specific effector cells (e.g., T lymphocytes) and/or differences in activated peritoneal versus spleen cells. Recent work by MacDonald et al. (1978a, b) indicates that a major portion of the destruction of the tumor cells is mediated by cytolytic T lymphocytes in experiments where phagocytic and nonadherent cells were removed with specific antisera and complement. This is in agreement with Strom et al. (1977) who found that cytotoxicity in rat cardiac allografts was mediated by T lymphocytes. In contrast, Roberts and Häyry (1976, 1977), using artificial sponge allografts, have found both T and non-T lymphocytes to be involved in the cytotoxicity. The morphological data presented here suggest that macrophages do become closely associated with the tumor cells and indicate the necessity for further investigation to delineate their role as possible effectors, helpers, or scavengers.

Although work to date with the multicellular spheroid has been focused on the sensitized allogeneic systems, other experiments indicate that similar infiltration and destruction of the spheroids occur in normal, nonsensitized allogeneic mice, although there is considerable alteration in the kinetics so that 6–8 days rather than 1–2 days are required for maximum destruction. Furthermore, preliminary work shows that the multicellular spheroid will also be useful for studies in syngeneic systems, so that responses to tumor-specific antigens can also be analyzed. Since the EMT6 mammary sarcoma is a tumor which elicits an immunological response (Rockwell, 1977; Rockwell et al., 1972) and, in vivo, solid EMT6 tumors are often extensively infiltrated by host lymphoid cells, the infiltration of multicellular spheroids by host cells may be closely analogous to the normal in vivo situation. Thus, multicellular spheroids provide an excellent model for studying the functional capacity of host cells within the microenvironment of a tumor and the modification of their activity by different cancer treatment modalities.

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