Journal of Supramolecular Structure
and Cellular Biochemistry 15:317-326 (1981)

Phenotypic Diversification of a Cultured Tumor Line as a Function of Substratum

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We have found that a murine hepatoma displays a considerable phenotypic diversification in culture, which depends upon the substratum utilized, and is manifested by the formation of multicellular structures of differing geometry: Monolayer on glass and plastic, thick multilayer pads on Gelfilm, and spheroids on agar and agarose. These multicellular morphological phenotypes were assayed without disruption to ascertain their antigenicity in vitro and their tumorigenicity in vivo and to obtain quantitative information on the effect of the spatial arrangement of the hepatoma cells upon the ability of each multicellular structure to interact, as a whole, with molecules and cells in its surroundings.

The antigenicity of the multicellular structures was determined with calibrated probes and a methodology that measures the total antigenicity, as well as antigenicity per unit of surface area. Antigenicity was found to differ in the following decreasing order: Monolayer on plastic > spheroids on agarose > spheroids on agar > multilayer on Gelfilm. At least part of these antigenic variants arise from different degrees of masking of the structures' surface determinants by a trypsin-sensitive material.

The multicellular phenotypes also differed in tumorigenicity. When assayed in syngeneic hosts under comparable conditions, agar-grown spheroids produced the fewest tumors, whereas Gelfilm-grown multilayers produced the most.

These two independent sets of data show that the various geometries that a tumor tissue is induced to acquire by the culture substratum are accompanied by a distinctive combination of surface and biological properties.

Key words: **culture substratum, influence on morphogenesis; mouse hepatoma, phenotypes in vitro; surface antigens, modulation by culture substratum; tumor malignancy in vivo, modification by preculture**

Cell proliferation in the embryo sets in motion a sequence of events that generates a dynamic microenvironment [1]. Newly formed cells emerge into a microecological niche that differs from the one in which their predecessors developed; the daughter cells contribute to the generation of yet another niche for their progeny. Moreover, it is likely that the environmental pressures acting upon the cells differ according to their position within the expanding supracellular structure – ie, the central or peripheral regions. Thus, morpho-

Received August 22, 1980; accepted February 23, 1981.

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genesis is the result of a complex series of signals delivered to the cell genome and metabolic machinery as its outer membrane comes into contact with surrounding molecules [1–9].

Studying these developmental phenomena involving cellular differentiation, in vitro, requires the use of culture media and substrata [1, 4, 7–14]. This requirement has brought about a growing emphasis upon the development of new techniques for manipulating cell lines as experimental models for morphogenesis. In addition, there is a need to understand the impact of these manipulations upon gene expression. Relatively little is known about the extent of diversification that a cell line can undergo if cultivated on different substrata. This is surprising, since it is well established that a) the interaction of cells with contacting structures plays a role in histogenesis and organogenesis, as mentioned above, and b) the culture conditions affect the attachment of cells to each other and to the substratum [9, 11, 15–20].

We report here our findings with a murine hepatoma subline that exhibits a considerable degree of phenotypic variability associated with culture substratum; ie, it forms different multicellular structures, depending upon which substratum is used. Our emphasis herein is directed toward the analysis of these multicellular structures, or cell-line phenotypes, with as little disruption as possible, to obtain quantitative information on the effects of the structures' geometry on their ability to interact with their surroundings. Thus, intact structures were assayed in vitro to measure their capacity to bind antibodies and in vivo to compare their potential for tumor production in syngeneic hosts.

These kinds of studies are needed to provide insights into the mechanisms that modulate the expression of surface molecules involved in the recognition events [1, 21] occurring primarily at the periphery of 1) tissues and organs as they are being modeled into their physiological geometry during growth and 2) tumors while invading adjacent tissues and metastasizing in preferential locations.

MATERIALS AND METHODS

Cell Line and Media

The cell line used in these studies was derived from the BW7756 hepatoma of C57L/J mice (Jackson Laboratories, Bar Harbor, ME). It expresses a reduced tumorigenic potential from the original HEPA culture line [22, 23] brought to the Division of Laboratories and Research for studies by Dr. Gerald J. Mizejewski. For this reason, we have designated it Albany–HEPA subline, or, for brevity in this work, Albany subline. The subline is maintained in number 3024 plastic culture flasks (Falcon Plastics, Cockeysville, MD) in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, and Division of Laboratories and Research, New York State Department of Health, Albany, NY) containing 10% fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Substrata

Albany cells were cultivated in RPMI 1640 with glutamine (Gibco) and supplements, as mentioned above, on five different substrata. These included the bottom of number 3024 plastic flasks, described above, and glass slide coverslips (both representing rigid, impermeable growth surfaces); a gelatin film, Gelfilm (Upjohn Co., Kalamazoo, MI) [24]; a 2–3-mm layer of 1% Bacto Agar (Difco Laboratories, Detroit, MI) [25–27]; and a 2–3-mm layer of 1% agarose (Cal-Biochem, San Diego, CA) on the bottom of a number 3024 flask (semirigid, permeable substrata).

Tumorigenicity Assays

The tumorigenic potential of Albany subline multicellular phenotypes grown over the various substrata described above was compared in C57L/J mice (Jackson Laboratories), where the original hepatoma arose. Cells forming a confluent layer on plastic were removed by a 1-min exposure to 0.05% trypsin (Difco Laboratories) and subsequent washing in three 10-ml volumes of serum-free medium. The trypsinized plastic-grown cells, pieces of Gelfilm covered with intact multicellular layers and multicellular spheroids grown over agar or agarose, were subcutaneously implanted into four respective groups of anesthesized mice. Cell quantitation for the Gelfilm and spheroidal phenotypes was done by counting the number of cells on equivalent-sized and -aged Gelfilm strips, or in equivalent numbers of spheroids, after trypsinization. Tumor incidence was recorded at regular intervals and autopsy was performed at death. Significance was determined by standard chi-square evaluations using a two-sided probability.

Preparation of Antiserum

Gelfilm-grown Albany subline cells were removed without trypsin and washed 3 times in serum-free RPMI 1640 with glutamine and antibiotics before being used to immunize adult female rabbits (Division of Laboratories and Research). The initial subcutaneous injection of 48×10^6 cells, without adjuvant, was followed by boosting injections at 5, 7, and 9 weeks. Additional boostings were administered at 4 and 8 months (70 and 30×10^6 cells, respectively). The reported results were obtained with serum drawn 41 days after the last injection. The antiserum was not preadsorbed, in order to preserve all specificities, because we desired to measure total antigenicity.

Comparison of Albany-Subline Phenotypes as Defined by Antiserum to Surface Antigens

The antigenic phenotype of each morphological variant was evaluated, by adsorption of the above-described antiserum and subsequent determination of remaining cytotoxic activity, using trypsinized, plastic-grown cells as targets. Prior to all adsorptions, the antiserum was inactivated at 56°C for 1 h. Three groups of adsorptions were run with different parameters in each as described in the paragraphs that follow.

Adsorption of antiserum with equal volumes of each phenotype. Albany-subline cells were removed from the plastic substratum by trypsinization as described above. Comparison of these trypsinized cells to cells removed from the plastic substratum by mechanical means showed no differences in adsorptive capacity at the dilutions tested (data not presented). Trypsinization did, however, yield cell suspensions with higher percentages of viability. Multicellular layers grown on Gelfilm were scraped off and gently aspirated. Multicellular spheroids grown over layers of agar or agarose were collected by centrifugation. Cell suspensions from each phenotype were separately washed 3 times in 10 ml of serum-free RPMI 1640 and pelleted by centrifugation at 130g for 10 min. For each 100 μl of pellet volume recovered, 100 μl of undiluted antiserum was added, and the pellets were resuspended. Adsorptions were run for 30 min at 20°C, followed by 30 min at 4°C. Antisera were recovered by centrifugation as above.

Adsorption of antiserum with equal numbers of trypsinized cells from each phenotype. Albany subline cells were removed from the plastic substratum by 1-min exposure to 0.05% trypsin. Cells growing on Gelfilm and in spheroids were released by the same exposure to trypsin and gentle aspiration. All 4 single-cell suspensions were washed in serum-free RPMI 1640 as above and counted, using 0.1% trypan blue exclusion to determine via-

bility. To each 66.8×10^6 cells present – ie, the number of cells found in 100 μl of packed, plastic-grown cells – 100 μl of undiluted antiserum was added. The pelleted cells were resuspended and allowed to adsorb as outlined above.

Adsorption of antiserum with equal adsorbant surface areas (EASA) of each phenotype. Using a calibrated ocular reticule, cell diameters were measured in several samples of trypsinized, plastic-grown cells in 32-μm divisions. Since the trypsinized single cells resemble a sphere, the surface area for each cell can be approximated by the formula πd^2 (where d = cell diameter). The total adsorbant surface area (ASA) was thus estimated to be 7.89 $cm^2/10^6$ cells. As shown in the previous section, there are 66.8×10^6 cells/100 μl of packed single cells, or 527.05 cm^2/100 μl packed single cells. This value allowed us to define a standard ASA/antiserum ratio of 1:1 as 527.05 cm^2 ASA/100 μl antiserum. A 1:25 dilution in serum-free RPMI 1640 was chosen as a standard because it was the highest dilution capable of producing 80% or more cytotoxic activity against trypsinized, plastic-grown target cells. To maintain the 1:1 ASA/antiserum ratio, with 1:25 diluted antiserum, the ASA value was divided by 25 to give 21.08 cm^2 ASA/100 μl, 1:25 diluted antiserum.

Albany-subline cells growing in plastic culture flasks were used for antiserum adsorption as soon as the cultures reached confluency. The ASA of the undisturbed single-cell layer was taken to be the same as the area of the growth surface in the flask.

Since the undisrupted multicellular spheroids grown over agar and agarose approximate spheres, the ASA for these structures was determined in a manner similar to that used for the trypsinized, plastic-grown cells. The diameters of intact spheroids were measured in several culture samples. A distribution of the diameters was established, and an estimate of the ASA was obtained.

Undisturbed multicellular layers used for antiserum adsorptions appeared to be uniform and flat. The ASA for these structures was approximated by determining the surface area of the Gelfilm strip upon which the layers were growing.

Having established these parameters, the ASA/antiserum ratio could be decreased in order to saturate the exposed antigenic determinants. In the plastic culture flasks, the growth area was limited to 25 cm^2, so increasing amounts of 1:25 diluted antiserum were added to the undisrupted, confluent cell layer. When intact multicellular spheroids grown over agar and agarose were used for adsorption, the volume of 1:25 diluted antiserum was held constant and the number of spheroids was decreased to obtain the desired ASA. Adsorptions using undisrupted multicellular layers growing on Gelfilm were done in a similar manner; ie, the volume of 1:25 diluted antiserum was held constant, while the ASA was decreased by using smaller sections of covered Gelfilm.

Testing of Adsorbed Antisera

Trypsinized, washed, plastic-grown Albany-subline cells were used as standard target cells at a concentration of 1×10^7/ml. Fresh guinea pig serum was diluted 1:2 in serum-free RPMI 1640 and used as a complement source. In the absence of antiserum, this complement had $<3\%$ cytolytic activity. Antisera adsorbed by equal volumes of intact multicellular phenotypes and equal cell numbers from these phenotypes (but not the antisera from the EASA series of adsorptions) were diluted in serum-free RPMI 1640 after completion of the adsorptions (as shown under Results) prior to use in the cytotoxic assay. One hundred microliters of target cell suspension, complement, and antiserum were mixed in each tube and incubated at 37°C for 1 h. Cytolysis was determined by 0.1% trypan blue exclusion, and values under 10% were considered to be negative (no cytolytic antibodies remaining).

RESULTS

Considerable morphological variation accompanied the growth of the Albany-subline on the different substrata used in this study (Fig. 1). These multicellular structures or morphological phenotypes interconverted to any one of the others when cultivated on the proper substratum (data not shown).

The Gelfilm phenotype was the most tumorigenic, and agar-grown spheroids were the least ($P < 0.05$). The remaining two phenotypes were in between, although the agarose-grown phenotype also tended to show low tumorigenicity as compared to Gelfilm (P very close to 0.05) (Table I). Tumor incidence did not change after day 28.

The antigenicity of the morphological phenotypes was first determined by adsorbing the antiserum with an equal volume of each phenotype. On this equal volume basis, the agar- and agarose-grown spheroids were less antigenic than the other phenotypes since more antibodies remained after incubation of the antiserum with the spheroids than after

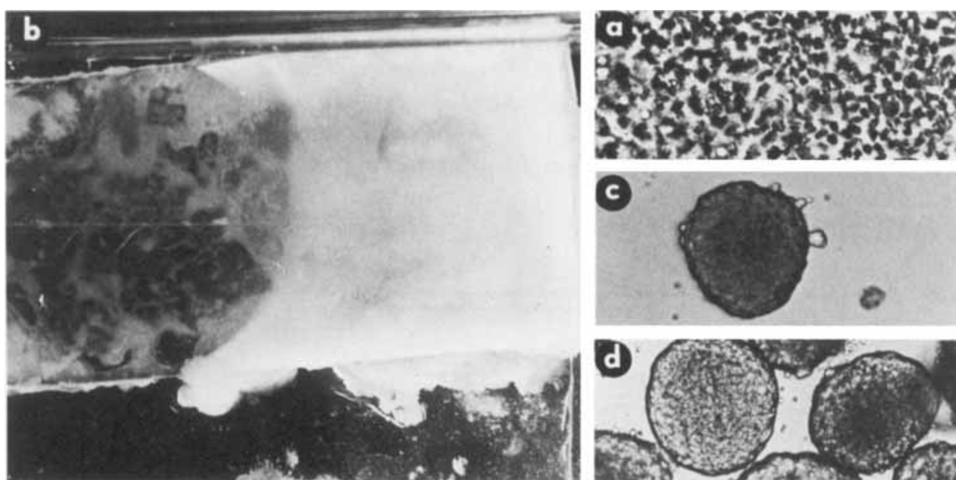

Fig. 1. Morphological phenotypes of the Albany subline: a) Cell layer on plastic (×100), b) thick multilayer structure on Gelfilm (×5), c) spheroids on agar (× 63), and d) on agarose (×63).

TABLE I. Tumorigenicity of the Hepatoma Subline Phenotypes

	Tumor incidence, days after inoculation[a]	
Phenotype grown on	14	28
Gelfilm	11/14 (79)[b]	14/14 (100)
Plastic	3/12 (25)[c]	10/12 (83)
Agarose	4/11 (36)	7/11 (64)
Agar	2/11 (13)[c]	5/11 (45)[c]

[a] 5×10^5 viable cells inoculated subcutaneously.
[b] Mice with palpable tumors/mice studied (percent of mice with palpable tumors).
[c] Different from Gelfilm-grown phenotype ($P = 0.05$).

incubation with plastic or Gelfilm phenotypes. The latter two phenotypes removed virtually all antibodies (Fig. 2). This difference between spheroidal and flat phenotypes most likely reflects the difference in exposed surface area available for adsorption, which is much smaller in spheroidal than in flat structures when equal volumes are compared.

When equal adsorptive surface areas from each phenotype were compared, plastic and agarose-grown phenotypes showed the highest antigenicity (adsorptive capacity) (Fig. 3). These two phenotypes could not be saturated with a 20-fold excess of antiserum; no antibodies were left unadsorbed even at an ASA/antiserum ratio of 1:20. In contrast, the Gelfilm-grown phenotype showed the lowest adsorptive capacity per unit area since it could be saturated with the smallest amount of antibody. At an ASA/antiserum ratio of 1:6, a significant amount of antibodies was left unadsorbed. The agar-grown phenotype also exhibited a low adsorptive capacity but required 2.5-fold more antibodies than the Gelfilm phenotype to become saturated. Considering the results shown in Figures 1 and 2, the following decreasing order of antigenicity among the phenotypes becomes apparent: phenotype grown on plastic > agarose > agar > Gelfilm.

The effect of trypsinization upon the various phenotypes is shown in Figure 4. The cells from the agarose phenotype showed a reduced antigenicity compared to the cells from the other phenotypes. This indicates that the agarose phenotype, which proved to be highly antigenic (Fig. 3), has its determinants more exposed to trypsin action than, for example, the Gelfilm phenotype. The latter probably has its determinants covered by a trypsin-sensitive material, hence its lower antigenicity as compared to the other phenotypes in the absence of trypsinization (Fig. 3) and its significant increase in antigenicity after trypsinization (Fig. 4).

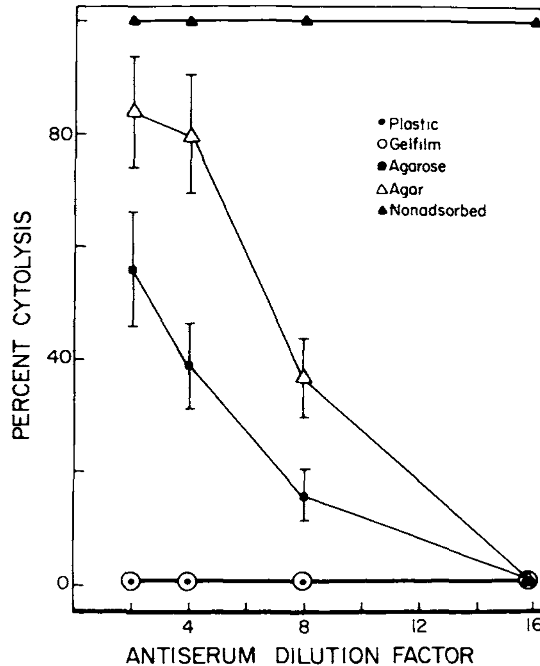

Fig. 2. Equal volumes of each undisrupted morphological phenotype were used to adsorb equal volumes of undiluted antiserum. Serial dilutions of these adsorbed antiserum samples were assayed for cytolytic antibodies in parallel with nonadsorbed antiserum using trypsinized, plastic-grown target cells (AM ± 2 SD; n = 3).

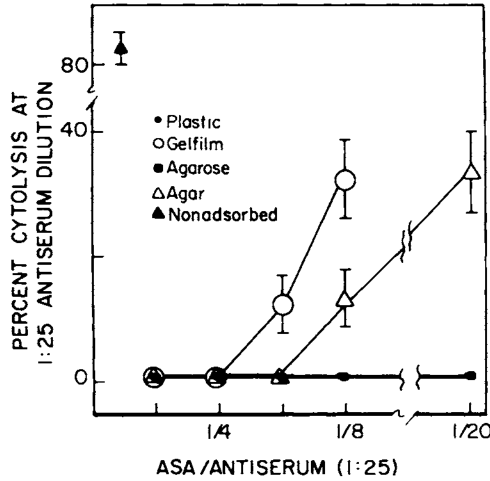

Fig. 3. Equal absorptive surface areas (ASA) of each undisrupted morphological phenotype were used to adsorb the standard antiserum. Prior to the adsorption procedure, the antiserum was diluted 1:25, which was the maximal dilution producing 80–85% cytolysis in repeated tests. Various ASA/volume of antiserum ratios were utilized in the adsorption procedure, as indicated. The adsorbed antiserum aliquots were assayed for cytolytic antibodies without further dilution in parallel with the nonadsorbed antiserum using trypsinized, plastic-grown target cells (AM ± 2 SD; n = 3).

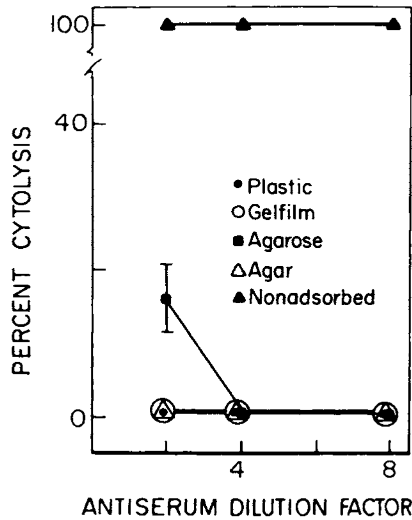

Fig. 4. Equal numbers of trypsinized cells from each morphological phenotype were used to adsorb equal volumes of the undiluted standard antiserum. Serial dilutions of the adsorbed antiserum aliquots were assayed for cytolytic antibodies as in Figure 2 (AM ± 2 SD; n = 3).

The derivation of the standard 1:1 ASA/antiserum ratio for nontrypsinized plastic-grown cells ensures that the surface area used for antiserum adsorption at the 1:1 ASA/antiserum level in Figure 3 is identical to that for the 1:1 packed cell/antiserum volume

ratio, which was used to adsorb antisera with trypsinized plastic-grown cells in Figure 2. We compared the adsorptive capacities of trypsinized and nontrypsinized plastic-grown cells with equal ASA values and found them to be identical to an ASA/antiserum ratio of 1:3 (data not presented), excluding the possibility that the masking protein was of fetal origin.

DISCUSSION

Data in this report demonstrate that the culture substratum can influence the supracellular organization or multicellular morphological phenotype of a mouse hepatoma grown in vitro. A distinctive combination of morphological phenotype, antigenicity, and tumorigenicity was observed for each substratum used. Antigenicity was measured by calibrated adsorptions of a standard antihepatoma antiserum with comparable samples of each morphological phenotype. This procedure was applied at two levels of sensitivity, depending upon the degree of dilution of the antiserum used for adsorption: Either undiluted or at the maximal dilution capable of producing 80–85% cytolysis in the presence of complement. Cytolytic antibodies remaining after adsorption were measured with standard trypsinized target cells grown on plastic and were selected on the basis of pilot experiments that showed that they are the most antigenic of all the phenotypes studied. These plastic-grown, trypsinized cells were the most efficient adsorbers of antibodies from our standard antiserum. We therefore assumed that these cells express all specificities that are present on the other phenotypes, as well as their own. Thus, the absence of lysis in the presence of an adsorbed antiserum was taken to indicate the absence of antibodies but not of the relevant antigenic determinant on the target cell. The results obtained confirmed this assumption.

Adsorptions were performed with equal volumes and equal surface areas (EASA) of the undisrupted multicellular structures and equal numbers of trypsinized cells from each one of these phenotypes. The results obtained with the former two, equal volume and EASA of multicellular structures, demonstrated the following decreasing order of antigenicity: Phenotype grown on plastic > agarose > agar > Gelfilm.

The results obtained with trypsinized cells from the phenotypes suggest that the low antigenicity of the Gelfilm- and agar-grown phenotypes, as compared to the other phenotypes, is due to masking of surface determinants by a trypsin-sensitive material. This material does not seem to be as prevalent on the agarose-grown spheroids, whose surface determinants appear to be directly exposed to trypsin action.

Since it is known that serum components in the culture medium are difficult to remove from the cell surface by washing [28], one has to consider the question of whether some of the results observed are due to antibodies directed toward fetal serum proteins. This possibility is ruled out in our system since the standard target cells for the assessment of cytolytic antibodies remaining after adsorption were trypsinized – ie, devoid of serum proteins. Thus, antibodies to these proteins, if present at all in our antiserum, had little significance and were irrelevant in the case where trypsinized cells were also used for adsorption. In the case where nontrypsinized cells were used for adsorption, the antifetal protein antibodies that might have been present in our antiserum were adsorbed out, together with the antibodies to membrane antigens, and therefore were excluded from the system prior to the assay for remaining antibodies.

The possibility still remains that some of the differences in antigenicity detected by means of calibrated adsorptions with nontrypsinized intact multicellular structures are due to differential masking of surface antigens by fetal serum components. If this were the case,

it would reinforce the conclusion that the various phenotypes do differ in surface properties: Antigenicity and affinity for fetal serum components. The latter point remains to be analyzed.

The mechanism by which the substratum generates diversification in our system might be similar to the one that generates spheroids in other tumor lines [27] and modulates mesenchyme spreading [29]. Most likely, this involves the degree of substratum permeability and surface physicochemical properties and medium modification [1, 4, 10–20] by substratum-derived products released into the culture fluid.

Taken together, our data indicate that the surface properties of a multicellular structure are modulated by its shape and that the reactivity of the cells that make up the surface of the structure is influenced by their own cell organization in space. This endogenous modulation is probably brought about by interactions between the outermost cells and those cells lying immediately underneath and so forth, down to the core of the structure. These regulatory interactions between contacting cell layers would be similar to the ones known to occur between a substratum and the cells grown on it [9, 11, 15–20, 30]. A further analysis of the antigenic specificities of the multicellular phenotypes should elucidate whether they are species-, tissue-, and/or tumor-specific and may also contribute to the clarification of their role in tumor immunity and determination of tumorigenicity in vivo. However, interpretation of the tumorigenicity data must take into account immunological as well as nonimmunological factors. For example, spheroidal phenotypes might have an immunological advantage since they expose a proportionally smaller surface to cytolytic antibodies and/or cells than do flat structures of the same volume. Although this seems to be the case in our model system, as suggested by the antigenicity measurements in equal volumes of spheroidal and flat multicellular structures, some exceptions are conceivable. For example, if the exposed segment of the outermost cells of spheroidal structures develop villi and/or other prolongations, the effective surface of the spheroids could be maximized and thus make them as accessible to antibody binding as flat structures of the same total volume but with a bald exposed surface. Moreover, nutritional limitations placed upon the inner core cells by the spheroidal symmetry may hamper tumor growth.

ACKNOWLEDGMENTS

We thank Dr. Joseph Leighton for his criticism, Dr. Gerald J. Mizejewski for supplying the original HEPA cell line, and Ms. Sally Hauser for her editorial comments.

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