DIFFERENTIAL GROWTH OF ENZYME-DISSOCIATED AND NON-DISSOCIATED RAT MAMMARY TUMOR TRANSPLANTS PLACED IN GLAND-FREE AND GLAND-CONTAINING MAMMARY FAT PADS

By

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ABSTRACT

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The major purpose of this study was to examine differential growth potential of primary transplants of carcinogen-induced rat mammary tumors. The secondary purpose was to determine the influence of normal mammary parenchyma on the outgrowth capabilities of those transplants Cell suspension of enzyme-dissociated tumors were used to determine whether more consistent results would be observed with dissociated transplants than with fragment transplants.

Eight of nine primary tumors induced by 7,12-dimethylbenz(a)-anthracene (DMBA) in Lewis rats were enzymatically dissociated and transplanted into gland-free and gland-containing inguinal mammary fat pads of syngeneic female hosts aged 9-12 weeks. Ten ul of cell inoculum containing an average of 1.5×10^7 cells/ml were injectd in each site. Tissue fragments of 1 mm^3 were similarly transplanted.

Whether transplanted as fragments or as enzyme-dissociated samples, similar patterns of heterogeneity in growth and morphology were found among tumors and within the same tumor.

When both types of transplants were used the growth behaviour of samples from two of eight tumors showed that fragments produced more

tumors than did dissociated transplants regadless of fat pad condition. In only one case dissociated transplants gave rise to more tumors than did fragments. In samples from two of eight tumors, no differences were found in tumor growth from fragments and enzyme-dissociated transplants. In another single case, all samples gave rise to tumors whether dissociated or not. Samples from the remaining two tumors showed low transplantability. Except for one instance, latencies for palpability were longer when transplants were subjected to enzyme dissociation than when they were not. Overall, no significant differences in tumor growth could be attributed to fat pad condition.

The finding that outgrowth development from tumors was inhibited following transplantation in gland-containing fat pads whereas outgrowth development from tumors was favored following transplantation in gland-free fat pads suggests that normal mammary parenchyma suppresses outgrowth development. Ovarian-dependence or ovarian-independence of samples from individual primary tumors played no role in determining latency, morphology, or outgrowth potential.

The growth capabilities of both enzyme-dissociated mammary tumors and mammary tumor fragments probably varied according to the lability of the respective tumor. The greater the lability, the greater the influence of the host microenvironment on growth outcome of the tumors.

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INTRODUCTION

Heterogeneity is a common characteristic of tumors, not only in tumors of the same type but also in individual tumors. Kobori and Outa (1979) pointed out that cells from chemically-induced gastric tumors varied in gastric, intestinal, and neuroendocrine secretory properties. They serially transplanted pieces of the tumors into isogenic hosts. The original tumors and those tumors resulting from transplantation were examined histologically, histochemically, and electron microscopically. Anatomical differences were observed between the original tumors and from the transplant-derived tumors. Electron microscopy revealed that both the original and transplant-derived tumors consisted of undifferentiated cells as well as gastric, intestinal, and squamous epithelial cell types and neuroendocrine cell types. Differential sensitivities to drugs have been observed in hepatomas (Barranco et al., 1978), in which the anticancer drugs bleomycin, adriamycin, 1-β-D-arabinofuranosylcytosine, hydroxyurea, 1-trans-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea were tested. The susceptibilities of cell lines derived from the original hepatoma ranged from little sensitivity to almost total resistance. Differential susceptibilities to retinoic acid-induced growth inhibition in melanomas were observed by Lotan (1979), who examined ten human cell lines including melanomas and breast carcinomas. These

lines were incubated in the presence of retinoic acid, and even though the cell lines had similar histopathological derivation, the degree of inhibition differed.

Variability in metastatic capabilities has been shown in fibrosarcomas and melanomas (Fidler and Hart, 1981; Fidler and Kripke, 1977; Hart and Fidler, 1981; Poste and Fidler, 1980) and in lymphomas (Schirrmacher et al., 1979). Cell lines of lymphomas (Chow and Greenberg, 1981) and transplanted tumor clones of chemically-induced fibrosarcomas (Schmitt and Daynes, 1982; Woodruff et al., 1982) vary in growth rates and antigeneic properties. Variant clones isolated from primary parental tumors differed in the ability to form subsequent tumors upon transplantation into sygeneic hosts. They also differed in antibody-binding. Other heterogeneous characteristics include differential hormonal responses. For example, human endometrial tumors vary in response to progesterone (Siracky, 1979). Patients with endometrial cancer were given progesterone therapy, and tissue samples were obtained both before and after treatment. The nuclear morphology of the tumor cells was examined to determine the secretory conversion of the endometrial cell population and differences were formed within the cell population of a single tumor. Transplanted prostatic carcinomas also vary in response to androgens (Isaacs and Coffey, 1981).

Isaacs and Coffey used a slow-growing and well-differentiated transplantable rat prostatic adenocarcinoma to demonstrate heterogeneity with respect to androgen response. Tumor growth stopped after castration in the male rats bearing these tumors but grew again after a lapse of time. They concluded that the new growth was not due

to environmentally-induced adaptation from androgen-dependence to androgen-independence but due to the mixture of both androgen-dependent and androgen-independent clones within the tumor. Variability in pigment production in melanomas has also been demonstrated in another example of heterogeneity (Fidler and Hart, 1981).

Mammary tumors of different etiologies have been shown to be heterogeneous in a number of biological properties. Human spontaneous tumors vary in estrogen receptor content and ploidy number (Bichel et al., 1982). Bichel found that patients with estrogen receptor positive tumors showed variable response to endocrine therapy. He suggested that the mosaic composition of tumors accounts for the differential response to treatment. Tumors also vary in their susceptibility to retinoic acid-induced growth inhibition possibly as a result of variations in the level of a binding protein (Lotan, 1979). Spontaneous mouse mammary tumors, from which cell lines or clones have been prepared, differ in karyotype (Dexter et al., 1978), epithelial characteristics such as dome formations, expression of mammary epithelial antigens, functional complexes, and cell polarity (Hager et al., 1981) They also differ in their sensitivities to such drugs as cyclophosphamide, methotrexate, and 5-fluoracil, which are commonly used in cancer therapy (Heppner et al., 1978). The ability of cell suspensions made from spontaneous tumors to clone in culture and, upon injection into host mice, to produce tumors is variable according to Soule et al. (1981). The evidence was provided from five isolates, obtained from primary mammary tumors and grown in suspension culture, which were then established in monolayer culture for characterization and placed back into suspension culture for recloning. Differences in

the ability of the isolates to form tumors were noted after inoculation into male, female, or female athymic nude mice.

Chemically-induced mammory tumors are often used as models to study human tumors. Transplantation studies of these induced tumors reveal variable outgrowth potential *in vivo* such as ductal outgrowths and hyperplastic alveolar nodules as well as tumors in gland-free mammary fat pads (Rivera and Vijayarhavan, 1982). Similar variations in outgrowth potential have been demonstrated using cultured pregnancy-dependent mouse mammary tumors (Aidells and Lee, 1978) and *in vitro* transformed mammary cells (Richards and Nandi, 1978) transplanted into mammary fad pads *in vivo*.

The importance of hormones in the initial appearance of the neoplasia and its subsequent development and control is well known (Costlow and McGuire, 1978; Hollander and Diamond, 1978; Huggins *et al*., 1959; Medina, 1981; Meites, 1972; Nandi, 1978; Rivera and Bern, 1982; Welsch and Meites, 1978). One parameter used to assess ovarian-dependence is the presence of estrogen receptors (Bichel *et al*., 1982; King *et al*. 1965; Mobbs, 1966) although it has been claimed that estrogen receptors are present in ovarian independent tumors as well (Boylan and Wittlif, 1975; DeSombre *et al*., 1976; MacFarlane *et al*., 1980). Thus, the presence of estrogen receptors in the tumor is not totally reliable in determining ovarian-dependence in that there may be variable response to ovarian ablation.

Most DMBA-induced tumors have been shown to be ovarian-dependent (Dao, 1969; Haslam, 1979; Rivera and Vijayaraghavan, 1982). However, tumors developing from the transplants of these tumors may respond by regressing or not respond at all after ovariectomy (Rivera and

Vijayaraghavan, 1982). First transplant generations, however, have been shown to vary little from the parent tumor in estrogen receptors and ovarian-dependence (Lee et al., 1978; Kim et al., 1960; Horn et al., 1976; Miller, 1980). After continued transplantation, the tumors progress from hormone-dependence to hormone-independence (Kim et al., 1960; Kim & Depowski, 1976; Horn et al., 1976; Miller, 1980).

Explanations to account for tumor heterogeneity remain speculative, but four merit consideration. One is the idea that heterogeneity is a reflection of the multiclonal origin of tumors. There is some evidence to support this theory. Byar et al. (1972) determined that 85% of the human prostates removed as cancerous were multifocal. This was done by a step-section histological technique showing several independent and anatomically distinct sites of carcinoma development. The development of tumors from multiple clones was also suggested by Pimm and Baldwin (1977), who reported antigenic differences in recurrent sarcomas after chemically-induced primary tumors had been removed. The primary and secondary sarcomas had different rejection antigens which suggests that the chemical inducer affects many cells, some of which may lie dormant until the parental mass has been removed. A similar finding was reported by Prehn (1970) in studies of chemically-induced tumors, where regional differences in antigeneic properties were found in the same primary tumor. Further support for the multiclonal origin of tumors comes from work of Woodruff et al. (1982), who suggested that it is the intraclonal interactions from the individual chemically-induced fibrosarcomas that caused the differential transplantability of antigeneically distinct clones. Different karyotypes in mouse mammary tumor clones (Dexter et

al., 1978) and in human spontaneous mammary tumors (Bichel et al., 1982) indicate multiclonal origin.

Another explanation for heterogeneity may relate to the possibility that even monoclonally-derived tumors or their progeny undergo phenotypic variation. Nowell (1976) proposed his now well supported theory of tumor development through clonal evolution: the tumor acquires genetic variability and is influenced by host selective pressures thereby accounting for tumor heterogeneity. As early as 1965, Linder and Gartler used glucose-6-phosphate dehydrogenase (G-6-PD) genetic markers to study uterine leiomyomas. They found that G-6-PD heterozygotes, producing both type A and B enzymes, have only single-enzyme phenotypes in their tumors. Samples of adjacent myometrium show both types of enzyme variants. The finding of only single enzyme phenotypes in the tumors studied indicate a single clonal origin despite the processes that favor transitions from precancerous lesions to heterogeneous and invasive neoplasms. Fiaklow (1972, 1974) made similar observations in various human female tumors using the same enzyme marker method. In 1973, Fiaklow studied Burkitt's lymphoma, and found that after remission, original malignant cell lines were observed in the early re-emergence of the lymphomas although other clones emerged at later recurrences. Thus, properties such as invasiveness or clonal variation may be acquired as a result of clonal evolution.

A third theory was proposed by Foulds (1975). In general, the theory states that tumors progress through step-wise independent changes in different properties giving rise to successive cell populations or clones within the tumor. Evidence in support of this theory is offered by Hager et al. (1978), who determined that after

serial transplantation of mouse mammary tumor cells lines from a single tumor, there was no correlation of behavior such as invasiveness and latency periods and growth rates to immune reactivity. These results support Fould's second rule of the independent progression of different characteristics in the same tumor. His third rule that progression is independent of growth was also supported by Hager et al. (1978), who reported that some of the malignant characteristics from their study appeared at different times during the development of the tumors. Kiang et al. (1982) looked at several biological markers of mammary tumors including hormone-dependency, progesterone receptors, polyploidy, and thymidine kinase activity. Hormone-dependence progressed towards hormone-independence but the other markers varied after every 4th and 6th generation of in vivo transplantation. These authors suggested that there are regulatory mechanisms within the tumors among the various cell populations that may allow old clones to give rise to new ones as the tumor progresses. This interpretation was also suggested by Woodruff et al. (1982).

Finally, a fourth consideration in accounting for tumor heterogeneity is the influence of the host microenvironment. The host milieu has been shown to affect the expression of several biological characteristics such as metastasis, growth rates, melanogenesis, and toxic agent sensitivities (Fidler, 1983). According to Fidler, selective processes for these biological actions are dependent on both tumor cell properties and the properties of specific organs. In general, the properties of human, frog, and mouse tumor cells to spread and colonize can be influenced by host modulation, which is to say that the host influence is local, at the site of the tumor lodgement,

according to Tarin (1983). More specifically, the host microenvironment was found to affect metastasizing capacities of lymphoma lines which may have been responsive to the inductive signals of the microenvironment (Schirrmacher, 1980) and in vitro transformed hepatic cell lines that were metastatic only in vivo (Talmadge et al., 1979) as well as the colonizing and metastatic properties of cell suspensions from a single mouse mammary tumor (Tarin and Price, 1982).

Recent work in our laboratory on DMBA-induced rat mammary tumors demonstrated that tumor fragments do are not always form tumors when transplanted into gland-free mammary fat pads. Normal-appearing ducts, hyperplastic alveolar nodules (HANs), and other dysplasias also form (Rivera and Vijayarhavan, 1982). There are several possible explanations. First, heterogeneous tumor cell populations may be present in the primary tumor which may account for variablity in outgrowth patterns. Second, there may be a potential for the tumor cell to undergo differentiation, and thus displaying different characteristics at different times. Third, because of phenotypic lability, a cell may be able to give rise to more than one type of outgrowth, which may
explain why fragments from the same tumor fail to consistently produce tumors only.

Furthermore, problems may be encountered in the use of tumor fragments as transplants. One problem is that zonal heterogeneity may make it difficult to obtain fragments that are representative of the tumor (Fidler and Hart, 1981). Cell numbers or content cannot be controlled in fragments. Another problem is that gland-free fat pads may favor ductal outgrowths and HAN proliferation over tumor growth.

An additional factor that might determine the type of outgrowths is the microenvironment at the site of transplantation. The gland-containing fat pad may be a the favorable site because of the improved growth of spontaneous and HAN-derived mammary tumors (Miller et al., 1981) and DMBA-induced mammary tumors (Vijayarhavan and Rivera, 1982).

Accordingly, the major purpose of this study is two-fold. The first is to examine whether differential growth capabilities of cell suspensions of primary mammary tumors, presumably homogeneous and enriched in tumor cells, will produce more consistent results than fragments of mammary tumors after transplantation. The second is to determine whether host factors such as the presence of the mammary gland parenchyma in the fat pad will play a role in influencing the growth of both fragment and enzyme-dissociated transplants.

The interesting findings provide evidence for the unique character of the individual primary tumors. The growth capabilities of both the enzyme-dissociated mammary tumors and the tumor fragments probably varied according to the lability of the respective tumor. The greater the lability, the greater the influence of the host microenvironment on the growth outcome of the tumor.

MATERIALS AND METHODS

Animals: Two sublines of Lewis strain rats, inbred by brother-sister matings, were used for this study. The original breeding pairs were obtained in 1976 from the National Institute of Health, Bethesda, MD, and from Simonsen's Laboratories, Gilroy, CA. Animals were maintained at constant temperature (27°C) with a controlled light cycle (12 Light-12 Dark). Food and water were administered ad libitum.

Induction of mammary tumors: Females were fed 20 mg of DMBA (Sigma Chemicals, St. Louis, MO) dissolved in sesame oil. The carcinogen was administered by gastric intubation in two 10 mg feedings, once when the rats were 45 to 50 days old, and again, one week later. Tumors developed beginning at 7 weeks after feeding. These were designated as primary tumors to distinguish them from those tumors derived from transplants.

Hosts: Most of the hosts used for transplantation studies were syngeneic virgin females from 9 to 12 weeks of age. Approximately one half of them had the fourth, fifth, and sixth pairs of their mammary anlagen and lymph nodes surgically removed by cauterization at 20 to 22 days of age (Rivera and Vijayarhavan, 1982). This procedure provided gland-free mammary fat pads to be used as transplantation sites. In addition, a small group of female hosts ages 22 to 27 weeks received transplants. Ten were gland-containing and seven were gland-free.

Another study utilized multiparous gland-containing (intact) hosts and gland-free hosts ages 50 to 52 weeks.

Controls: A group of animals both gland-free and intact were subjected to the surgery as controls to assure that any resulting dysplasias were not the result of surgical procedures. For the non-dissociated fragment studies, control animals had a small incision placed in the fat pad but without transplant. Three were intact and three were gland-free. For the dissociated transplant studies, three intact and four gland-free control animals received 10 ul of medium 199 (Grand Island Biological Co., Grand Island, NY).

Transplantation of mammary tumors: Ten randomly-selected primary tumors (1.5-1.8 cm diameter), each from a different rat, were excised under sterile conditions. With the use of the stereomicroscope, 1 mm cubes were cut from each tumor, excluding the capsule, and placed on saline-saturated filter paper in a petri dish. Tumor samples were transplanted singly into the right and left gland-free or gland-containing fat pads. Unless otherwise indicated, the ten tumors were designated by capital letters. Tumors A-H were transplanted into 9 to 12 week nulliparous hosts, and tumor I into 22 to 27 week hosts. Tumor J was transplanted into 9 to 12 week gland-containing nulliparous hosts, 50 to 52 week gland-containing multiparous hosts, and into 50 to 52 week gland-free nulliparous hosts. An established ovarian-dependent line, ST29 (4th and 6th generations) and an ovarian-independent mammary tumor line T41-8 (48th generation) were transplanted to compare the behavior of mammary tumor lines to that of primary tumors. Corresponding samples of all the tumors were fixed in Carnoy's solution for histological evaluation.

Preparation of cells for transplantation: In eight of the ten tumors studied, cell preparations were made by a modification of the method of DeOme et al. (1978). Modifications were time variations and slight procedural changes. Minced tumors were incubated in 0.1% collagenase and hyaluronidase (Sigma Chemicals, St. Louis, MO) in medium 199 at 37°C and hand-shaken every 10 minutes in a shaker bath for approximately 105 minutes instead of 90 minutes. The suspensions were placed in disposable 50 ml centrifuge tubes and centrifuged for 10 minutes at 1600 rpm. Subsequent centrifugations were done in the same manner. The pellet was resuspended in 1.25% pronase (45,000 PUK/g Calbiochem, Los Angeles, CA) in medium 199 for only 5 minutes instead of 15 minutes. Washings were done in cold medium 199 in a cold (4°C) room. Suspensions were filtered twice through 10 μm Nitex which was cut to fit a 22 mm Millipore Swinnex attached to a 20 ml syringe held in ice. Trypan Blue (0.5% in 0.85% NaCl) staining indicated 80% epithelial cell viability. Cell preparations were resuspended in medium 199 to a final concentration averaging 1.5×10^7 cells/ml.

Injection procedure: Using a Hamilton syringe number 705, 10 μl of the cell suspension was injected into the right and left gland-free and/or gland-containing inguinal mammary fat pads. Suspensions were mixed frequently and held in ice. The syringe was washed with cold 0.85% saline between injections.

Evaluation of Latency, Growth Potential, and Ovarian Dependency: Ten days after transplantation, and every four days thereafter, the mammary fat pads were palpated for tumors. Tumor latency was determined to be the number of days that elapsed between transplantation and initial palpability of the tumor. Tumors were allowed to grow up to

2.5 cm in diameter before the experiments were terminated. Only those tumors that were palpable prior to termination were considered to have grown, and samples of all tumors were processed for histological examination. If there were no palpable growths, animals were terminated between the 15th and 16th week after transplantation and the fat pads removed. Fat pads were spread on filter paper and fixed in buffered 10% formalin. After fixation, papers were removed and the fat pads were defatted in acetone, dehydrated in alcohol, stained with hematoxylin, stored in methyl salicylate for later evaluation of outgrowths such as local takes, small tumors, ductal, or hyperplastic alveolar nodules.

To evaluate the ovarian dependency of the tumors, the hosts were selected at random for bilateral ovariectomy. The ovaries were removed through dorsal slits in the body wall when the tumors reached 1.5 to 2.0 cm in diameter. Starting 7 days after ovariectomy, changes in size were recorded. Following conventional criteria, tumors were considered ovarian dependent if the size was reduced by 50% within 2 to 3 weeks after surgery. Those tumors that continued to grow were examined at autopsy for viability. If the tumors showed less than 50% viable epithelium, they were classified as ovarian dependent. If there was continued growth and greater than 70% viability, the tumors were considered ovarian-independent.

Statistics: Mean latencies were compared using the Mann-Whitney U non-parametric statistic. Percentages were compared using Arc Sin Percentage transformations.

RESULTS

I. Differential Growth Potential of Randomly Selected Primary Tumor Transplants: Non-Dissociated and Enzyme-Dissociated Cells.

A. Overall recovery.

Recovery of both dissociated and non-dissociated transplants refers to all local takes, tumors, ductal outgrowths, and tumor-ductal combinations. In general, the non-dissociated fragments showed significantly higher takes than their dissociated counterparts. The dissociated transplants took better in the absence of the gland than in the presence of the gland (Table 1,2). There was no indication of recoverable outgrowths in the sham-operated hosts.

B. Specific types of recoveries.

Local takes were those transplants confined to the site of transplantation which did not give rise to palpable tumors or mammary outgrowths (Figure 1). Fragment transplants remained localized significantly more frequently in the intact fat pad than in the gland-free pad. Recoveries of the localized fragments were also slightly better compared to the recoveries of dissociated transplants in the intact fat pad. Overall recovery of dissociated transplants were lower than that of the non-dissociated fragments.

In the evaluation of tumor growth, only those transplants which were palpable prior to termination were considered tumors. After

termination, further classification was based on the presence or absence of other types of outgrowths associated with the tumor.

The primary tumors used as sources for the transplants varied in their ability to give rise to palpable tumors. The results of samples from two of the eight tumors (F and G), where both non-dissociated and dissociated transplants were used, showed that non-dissociated fragments gave rise to significantly more palpable tumors than did their cellular counterparts irrespective of the condition of the fat pad. However, with samples from tumor B, the dissociated transplants gave rise to more tumors than did the non-dissociated fragments or dissociated transplants. The two extremes in growth behavior occurred with tumor D, whose transplants gave rise to 100% tumors irrespective of the type of transplant or condition of the fat pad, and with tumors A and C from which fragment transplants gave rise to only 5% and 14% tumors, respectively, whereas dissociated transplants developed no tumors at all. Results are summarized in Table 1 and 2.

C. Potential of tumor fragments and dissociated transplants to form palpable tumors in intact and gland-free fat pads.

With regard to fragment transplants, samples from two of the nine tumors (B and H) grew to palpable size in the intact fat pad, 29% and 70% respectively, whereas only 0 and 30% of the dissociated samples grew in the gland-free fat pad. Samples from three of the nine (D,F,G) grew equally well in both types of fat pads (70-100%), whereas samples from four of the nine (A,C,E,I) grew poorly regardless of the type of fat pad (<20%).

Table 1. Differential growth capabilities of non-dissociated and dissociated tumor transplants in gland-containing fat pads.

a. NON-DISSOCIATED	TUMOR DESIGNATION									
	A	B	C	D	E	F	G	H	I	TOTAL
LOCAL TAKES	4	6	6		4	1			3	24
OUTGROWTHS										
Palpable Tumors		4	2	10		7	7	7	1	38
Tumors & Outgrowths @	1				1		1			3
Ductal/HAN								1		1
OTHER		1								1
TOTALS										
# Takes/# samples transplanted (%)	5/10 (50)	11/14 (79)	8/12 (67)	10/10 (100)	5/8 (38)	8/10 (80)	8/8 (100)	8/10 (80)	4/10 (40)	67/92 (73)

Table 1 (cont'd.)

b. DISSOCIATED

	TUMOR DESIGNATION									
	A	B	C	D	E	F	G	H	I	TOTAL
LOCAL TAKES		1	2			1	1	2		7
OUTGROWTHS										
Palpable Tumors		8		10				5		23
Tumors & Outgrowths							1	2	2	5
Ductal/HAN										0
OTHER		1								1
TOTALS										
# Takes/#samples transplanted (%)	0/12 (0)	10/14 (71)	2/14 (14)	10/10 (100)	0/0 (0)	1/12 (8)	2/10 (20)	9/10 (90)	2/10 (20)	36/92 (40)

Table 2. Differential growth capabilities of non-dissociated and dissociated tumor transplants in gland-free fat pads.

a. NON-DISSOCIATED	TUMOR DESIGNATION									
	A	B	C	D	E	F	G	H	I	TOTAL
LOCAL TAKES	2	1	1		2	2	2	5		15
OUTGROWTHS										
Palpable Tumors				10		6	8	2	1	27
Tumors & Outgrowths @					3	2		1	2	8
Ductal/HAN		8	4		3				1	16
OTHER	1									1
TOTALS										
# Takes/# samples transplanted (%)	3/12 (25)	9/14 (64)	5/14 (86)	10/10 (100)	8/8 (100)	10/10 (100)	10/10 (100)	8/10 (80)	4/6 (67)	74/94 (79)

Table 2 (cont'd.)

b. DISSOCIATED

	TUMOR DESIGNATION									
	A	B	C	D	E	F	G	H	I	TOTAL
LOCAL TAKES			2			1	1	1		5
OUTGROWTHS										
Palpable Tumors		12		12			3	5		32
Tumors & Outgrowths								1	4	5
Ductal/HAN	2		2				1	1	2	8
OTHER	3	1				1	1			6
TOTALS										
# Takes/#samples transplanted (%)	5/12 (42)	13/14 (93)	4/14 (29)	12/12 (100)	0/0 (0)	2/12 (17)	6/10 (60)	8/10 (80)	6/8 (75)	56/92 (61)

With regard to dissociated transplants, samples from three of eight tumors (A,C,F) failed to produce tumors irrespective of the fat pad condition. On the other hand, transplants derived from tumors B,D,G, and H showed the same growth capability in both the gland-free and the intact fat pads. With tumor I transplants, there was a significant increase in tumor development in the gland-free fat pad than in the intact fat pads.

Considering tumors separately from other outgrowths, more tumors developed from fragments placed into the intact (61%) than in the gland-free fat pad (57%). In comparing fragment to enzyme-dissociated transplants in the intact fat pad, there was no significant difference in tumor development. More tumors developed from dissociated transplants (66%) than from fragments (57%) when placed in gland-free fat lads. The interesting finding was that when the total percentages of all tumors that grew were considered, there were no significant differences among the groups with respect to the type of transplant or the condition of the fat pad (Table 3).

Other types of outgrowths were normal-appearing ducts and hyperplastic alveolar nodules (HANs). The gland-free fat pad favored ductal proliferation especially from the fragments. Ductal outgrowths and HANs were also observed in combination with tumors, particularly in the gland-free fat pad. However, ductal outgrowths or HANs were not observed in the absence of tumors when non-dissociated or enzyme- dissociated transplants were placed in gland-containing fat pads except for one instance only; a fragment from tumor H gave rise to a small outgrowth (Figure 2). Both dissociated and non-dissociated transplants gave rise to combinations of tumors plus ductal/HAN combinations

Table 3. Summary: Comparison of the total number of tumors developing from both non-dissociated and dissociated transplants in gland-free and gland-containing fat pads.

	# of tumors/# transplants recovered (%)	
	Non-dissociated	Dissociated
+ gland	41/67 (61)	28/36 (78)
- gland	42/74 (57)	37/56 (66)

Statistic: Arc sin percentage transformation.

(Figures 3-6). When ducts or HANs formed without tumors, the types of growths ranged from small local ducts, ducts filling the fat pad, small and large HANs, to various ductal/HAN combinations (Figures 7-12). The type of outgrowth that developed was not influenced by the type of transplant, i.e. dissociated or non-dissociated.

There were some variants observed which did not fit into any previously-defined category. One was a floral cluster-like outgrowth formed from some of the fragments placed into gland-free fat pads (Figure 13) and from enzyme-dissociated transplants placed in the intact fat pad (Figure 14). Another variant was the formation of cell clusters as a result of cell suspensions of the dissociated transplants placed into gland-free fat pads (Figure 15). Outgrowth variants are summarized in Table 4.

D. Comparison of tumor latencies.

Latencies were determined for palpable tumors prior to termination. Of the transplants from the ten primary tumors, those from six gave rise to palpable tumors (B,D,F,G,H,J). Tumor J will be considered separately because the experimental protocol differed from tumors A-I. The mean latencies (14.3 ± 6.4, 10.8 ± 2.4 days) of tumors arising from non-dissociated transplants were significantly less than those (32.3 ± 11.6, 28.6 ± 9, 77.2 ± 9 days) of tumors from the dissociated transplants. However, in one instance (Tumor H), the mean latency was longer for non-dissociated transplants than dissociated transplants in the gland free fat pad ($88 \pm 15.4 > 52.0$ days). Results are summarized in Table 5.

Table 4. Summary: Comparison of growth potential of both dissociated and non-dissociated transplants in both gland-free and gland-containing fat pads.

Type of outgrowth		Non-dissociated	Dissociated
LOCAL	+ Gland	37	19[a]
	- Gland	20	9[b]
		a	c
TUMORS ONLY	+ Gland	57	64[c]
	- Gland	36	57[b]
		a	c
TUMORS & OUTGROWTHS	+ Gland	4	14
	- Gland	22	9[a]
		b	c
OUTGROWTHS DUCTAL/HAN	+ Gland	2	0[c]
	- Gland	20	14[c]
		b	b
OTHER	+ Gland	2	3[c]
	- Gland	2	11[b]
		c	b

Numbers represent the percentage of those recovered.
All statistics were done by Arc sin percentage transformations.
[a]significant at $p<0.05$
[b]significant at $p\leq 0.02$
[c]not significant

Table 5. Comparison of mean latencies of tumors from fragment and cellular transplants in gland-free and gland-containing fat pads.

	Tumor Designation				
	B	D	F	G	H
- gland ND vs D	-	14.3 ± 6.4 < 32.3 ± 11.6[b]	-	57 ± 17.6 < 77.2 ± 9[a]	88 ± 15.4 > 52[a]
+ gland ND vs D	-	10.8 ± 2.4 < 28.6 ± 9[b]	-	-	57 ± 24.5 ∿ 80
non-dissociated + gland vs - gland	-	10.8 ± 2.4 ∿ 14.3 ± 6.4	37.6 ± 8.9 ∿ 48.5 ± 15.6	51 ± 11.6 ∿ 57 ± 17.6	57.7 ± 24.5 ∿ 52
dissociated + gland vs - gland	48.3 ± 11.2 ∿ 45.6 ± 16.1	28.6 ± 9 ∿ 32.3 ± 11.6			80 ∿ 88 ± 15.4

mean latency in days ± standard error
- = no comparison because tumors did not grow
∿ = no significant difference
a = significant at p = 0.05
b = significant at p<0.05
Statistics used: Mann Whitney U
ND = non-dissociated
D = dissociated

E. Ovarian-dependency

Tumors were considered ovarian-dependent if > 50% regression was apparent two weeks after bilateral ovariectomy. In several instances, although gross tumor size continued to increase, only 30-50% of the tumor mass was viable at autopsy. The main finding was that variable responses of tumors derived from the same primary tumor were the rule, and the type of transplant or the fat pad condition was not a factor in hormone-responsiveness. Results are summarized in Table 6.

F. Influence of age, parity, and ovariectomy on tumor growth from transplants from tumor J.

Non-dissociated pieces from primary tumor J were placed in multiparous hosts of 50 to 52 weeks of age, nulliparous gland-free hosts of the same age, and in nulliparous 9-12 week hosts with intact mammary glands. There was 100% tumor development in the three groups. Multiple tumors and invasiveness (growth through the body wall) were apparent in several of the animals regardless of age, parity, or fat pad condition (Figures 16-20). A second generation of tumor J was prepared in nulliparous 9-12 week intact hosts. Tumor growth was again 100% but without any indication of multiple tumors or invasiveness. The latencies in all instances were less than twenty days irrespective of age, parity, or host fat pad condition. Results are summarized in Table 7.

Table 6. Response of transplanted tumors to ovariectomy.

	Tumor														
	B			D			F			G			H		
Number samples tested per tumor	11			16			4			6			3		
Number samples for each response	U	PR	R	U	PR	R	U	PE	R	U	PR	R	U	PR	R
	4	6	1	0	8	8	0	0	4	1	4	1	1	2	0

U = unresponsive (>70% of tumor viable)
PR = partial responsiveness (50 - 70% of tumor viable)
R = responsive (50% or less of tumor viable)

Table 7. Influence of age, parity, and ovariectomy on the growth of tumor J.

a. Growth and hormone dependence

Number of palpable tumors*/ number of samples transplanted (%)		Response to ovariectomy 2 tumors tested/category
9-12 weeks + gland nulliparous	8/8(100%)	2 responsive
50-52 weeks + gland multiparous	10/10 (100%)	1 responsive 1 partially responsive
50-52 weeks - gland nulliparous	8/8 (100%)	1 partially responsive 1 unresponsive
9-12 weeks + gland nulliparous 2nd generation	6/6 (100%)	2 responsive

* These tumors were unusual in that each site contained more than one tumor and were invasive.

b. Latencies

50-52 weeks - gland vs + gland multiparous	18 ± 4.2	14.2 ± 4
+ gland 9-12 wk vs 50-52 wk multiparous	11.6 ± 3.2	14.2 ± 4
+ gland 9-12 week 1st generation vs 2nd generation	11.6 ± 3.2	15.8 ± 7

= no significant difference
mean latency in days ! standard error

II. Variability in Growth Patterns and Morphology of Individual Primary Tumor Samples.

A. Variability of growth patterns is not affected by type of transplants (non-dissociated vs. dissociated) or fat pad condition.

Variable growth patterns were apparent from tumors arising from different primary tumors and in tumors derived from the same primary tumor.

All tumors derived from primary source B steadily increased in size over time once they became palpable.

However, all tumors derived from C were undetectable until the time of termination. The final palpation revealed the presence of small tumors in both the gland-free and gland-containing fat pads.

Samples from tumor D had short latencies of less than 35 days. The pattern of growth showed a steady increase in size over time. Upon termination, despite the solid appearance of the tumors, a high percentage of tumors was necrotic. The proportion of necrotic to viable tissue ranged from 30 to 70%. A second generation of tumors from tumor D showed similar results. A third transplant generation was impossible to initiate because of extensive necrosis of tumors from the second generation.

Of the transplants from tumor F that developed into tumors, seven either grew and spontaneously regressed or remained static. Of those tumors which regressed in size, some were no longer palpable in the living hosts but were recovered at termination. Four more tumors grew such that ovariectomy was possible and these four completely regressed after ovariectomy.

Transplants from tumor G gave rise to sixteen tumors out of thirty-eight transplants. All increased in size rapidly once they became palpable and were solid tumors without other outgrowths. These were in combination with ductal outgrowths in the gland-free fat pad. Growth of two other tumors was observed as slightly and gradually increasing in size over time.

Tumors derived from tumor H showed variable growth patterns. Nineteen small tumors developed with very little size variation throughout the experimental period. Four of the nineteen were associated with other outgrowths. In addition to the nineteen, four tumors steadily increased in size over time.

B. Morphological variations of tumors were not affected by type of transplant or fat pad condition.

All of the primary tumors used had common adenocarcinoma features such as well developed tubule or papillary formation or disorganized cell masses (Young et al., 1963). Random samples were taken from the primary tumors (Figures 21-23). Common patterns plus variations were noted in tumors arising from both non-dissociated and dissociated transplants. Variations were observed from rat-rat, tumor-tumor, and area-area within the same tumor (Figures 24-27). Tumor C was the only one whose transplants consistently produced tumors in a similar pattern observed in the parent tumor (Figures 21, 28-31). The same variability in morphological patterns was observed in tumors from both non-dissociated and dissociated transplants (Figures 32-35).

III. Tumor Growth Capabilities from Ovarian-Dependent and Ovarian-Independent Lines.

Previous studies in our laboratory invariably showed that ovarian-dependent tumor fragments gave rise to more palpable tumors in the gland-containing fat pad than in the gland-free (Vijayarhagvan and Rivera, 1982). Accordingly, fragments from an ovarian-dependent tumor line were placed in gland-containing fat pads only, but enzyme-dissociated transplants were placed in fat pads with and without the normal gland.

The ovarian-dependent line, ST29 (4th and 6th generations) developed only tumors in 60-100% of the transplants (Table 8). No significant differences were found among the groups with regard to the type of transplant or condition of the fat pad. The latencies were the same for the dissociated transplants in both the intact (5.9 $\pm$ 10.9, 62 $\pm$ 20 days) and the gland-free (6.7 $\pm$ 11.6, 59 $\pm$ 8.6 days) fat pads. However, the 4th generation of the dissociated transplant tumors had a longer latency (59 $\pm$ 10.9 days) than did the tumors from the non-dissociated fragments in the intact fat pad (20 $\pm$ days) (Table 8). Bilateral ovariectomy of the hosts bearing tumors supported the ovarian-dependent status in that size regression was apparent after two weeks.

The ovarian-independent line T41-8(48th generation) non-dissociated fragment transplants produced 100% tumors in both intact and gland-free fat pads with mean latencies of approximately two weeks. None of the dissociated transplants gave rise to tumors. There was evidence of local takes in only two instances. The tumors from the non-dissociated transplants showed neither regression nor necrosis after ovariectomy.

Table 8. Growth potential of transplants from ovarian dependent mammary tumor line ST29

	Number of tumors/number of samples transplants (%)		
	Generation 4	Generation 6	Totals
+ gland non-dissociated	9/4 (64) $\bar{X}$= 20 ± 6.1	9/10 (90) $\bar{X}$ = 74 ± 18	18/24 (75)
+ gland dissociated	10/10 (100) $\bar{X}$ = 59 ± 10.9	16/20 (80) $\bar{X}$ = 62 ± 20	26/30 (87)
- gland dissociated	10/10 (100) $\bar{X}$ = 67 ± 11.6	6/8 (75) $\bar{X}$ = 59 ± 8.6	16/18 (89)

$\bar{X}$ = mean latency for tumors in days.
Statistic: Arc sin percentage transformation

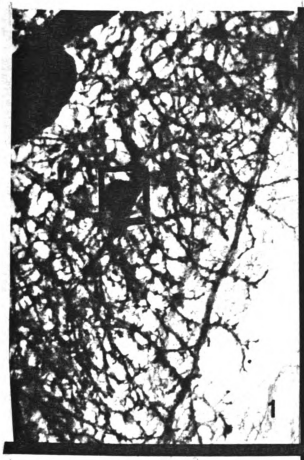
FIGURES

Figure 1 Example of a local take of a fragment placed into a gland-containing fat pad from primary tumor B 10x

Figure 2 Fragment from primary tumor H placed into a gland-containing fat pad. Note minimal outgrowth. 20x

Figure 3 Fragment from primary tumor C placed into a gland-free fat pad. Note alveolar and ductal outgrowth. Blank space indicates the site of tumor removal. 4x

Figure 4 Histology of tumor from Figure 3. Note secretory appearance. 400x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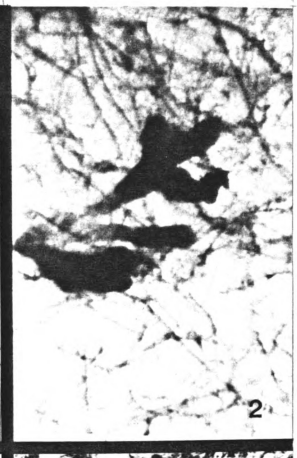
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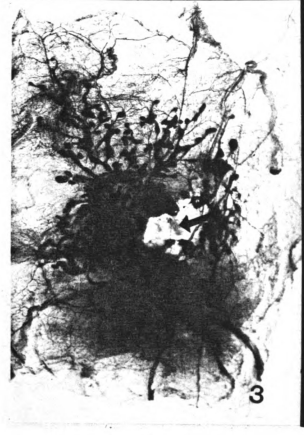
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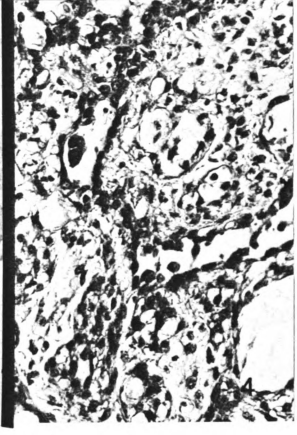
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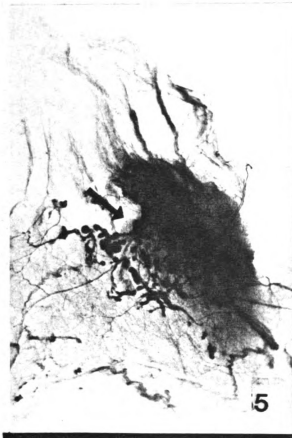
Figure 5 Cells from enzyme-dissociated tumor H placed into a gland-free fat pad giving rise to tumor and alveolar and dictal outgrowths. 4x

Figure 6 Histology of tumor from Figure 5. Note comedo structure and prominent stroma. 200x

Figure 7 Small ductal outgrowth from a gragment of primary tumor B placed into a gland-free fat pad. 10x

Figure 8 Large ductal outgrowth filling the fat pad resulting from a fragment of a primary tumor B placed into a gland-free fat pad. 4x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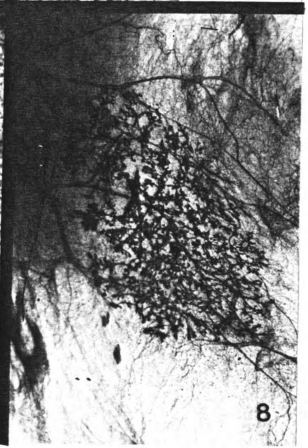
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Figure 9 Ductal outgrowth from cells of enzyme-dissociated tumor A placed into a gland-free fat pad. Note terminal end buds. 10x

Figure 10 Small HAN outgrowth from a fragment of primary tumor B placed into a gland-gree fat pad. 10x

Figure 11 Extensive HAN outgrowth resulting from cells of enzyme-dissociated tumor I placed into a gland-free fat pad. 10x

Figure 12 Combination of ductal and HAN outgrowths resulting from cells of enzyme-dissociated tumor I placed into a gland-free fat pad. 10x


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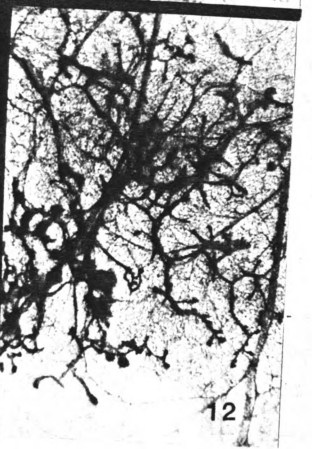
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Figure 13 Fragment from primary tumor B placed into a gland-free fat pad showing a floral-like cluster. 20x

Figure 14 Cells from enzyme-dissociated tumor A placed into a gland-containing fat pad showing a floral-like cluster. 20x

Figure 15 Cell clusters resulting from enzyme dissociated tumor A placed into a gland-free fat pad. 20x


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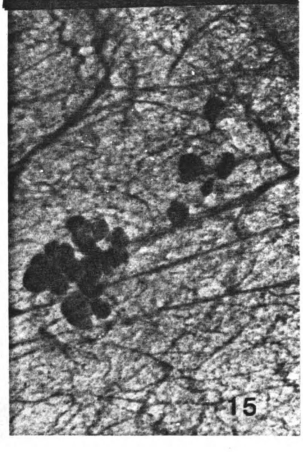
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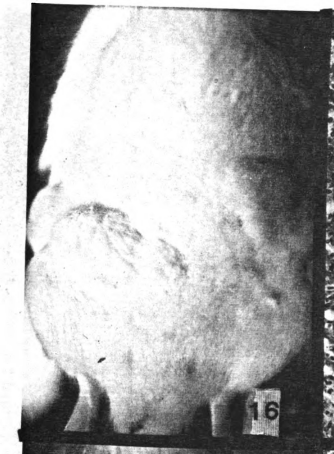
Figure 16 External view of rat mammary tumors from primary tumor J. Fragment placed into the fat pad of 50-52 week multiparous hosts. Note the multiple tumors seen as bumps on surface of skin.

Figure 17 Histologic sample of main tumor mass from Figure 16. 200x

Figure 18 External view of rat mammary tumors from primary tumor J. Fragment placed into the fat paid of 9-12 week hosts. Multiple tumors evident.

Figure 19 Histologic sample of main tumor mass from Figure 18. 200x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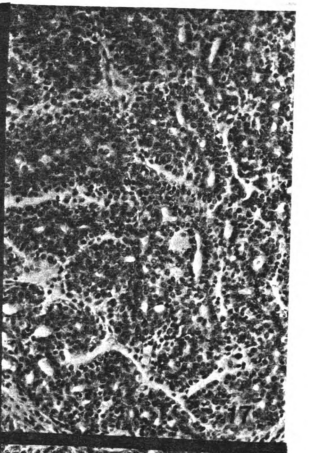
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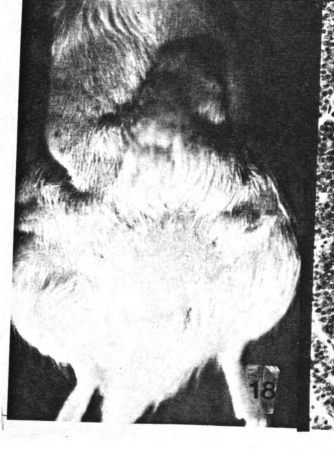
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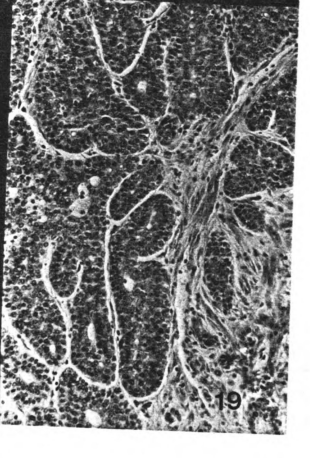
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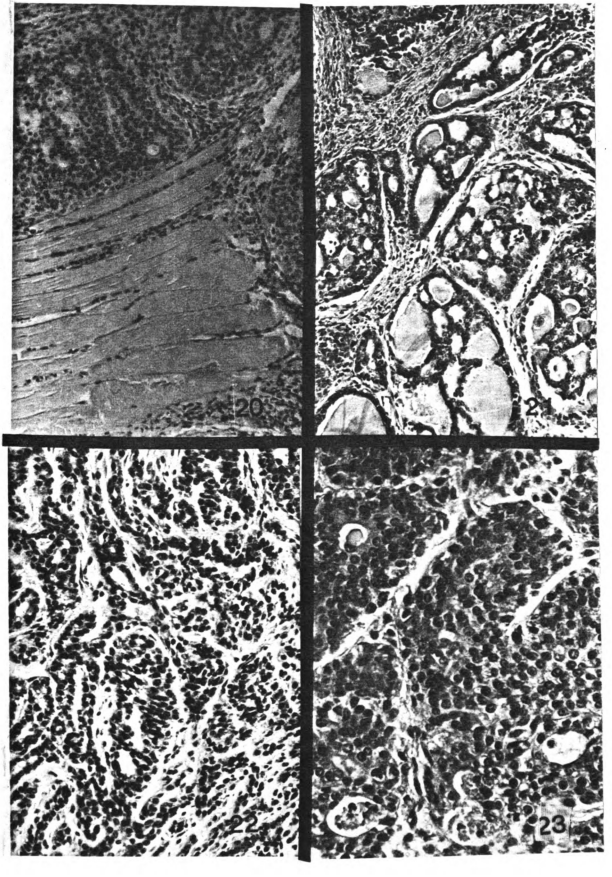
Figure 20 Multiple tumors from primary tumor J were invasive as shown by the presence of body wall muscle surrounded by tumor tissue. 200x

Figures 21-23 were taken to show differences were taken to show differences in morphology among primary tumors.

Figure 21 A random tissue sample taken from primary tumor C. 200x

Figure 22 A random tissue sample taken from primary tumor D. 22x

Figure 23 A random tissue sample taken from primary tumor H. 400x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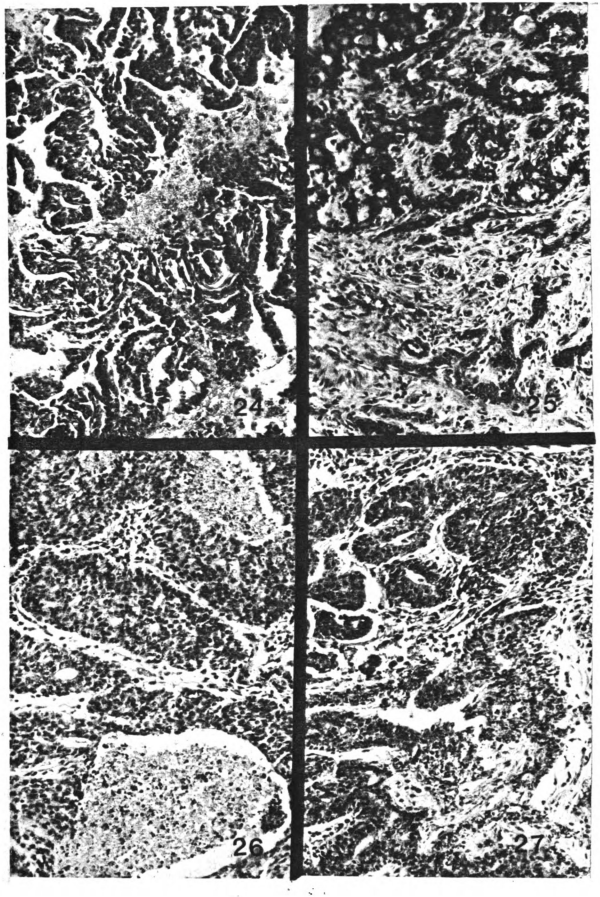
All of the following transplants were taken from primary tumor D. Note the morphological pattern variation.

Figure 24 Dissociated transplants placed into a gland-free fat pad. 200x

Figure 25 Fragment transplant placed into a gland-containing fat pad. 200x

Figure 26 & 27 Portions of the smae tumor derived from a fragment transplanted into a gland-containing fat pad. 200x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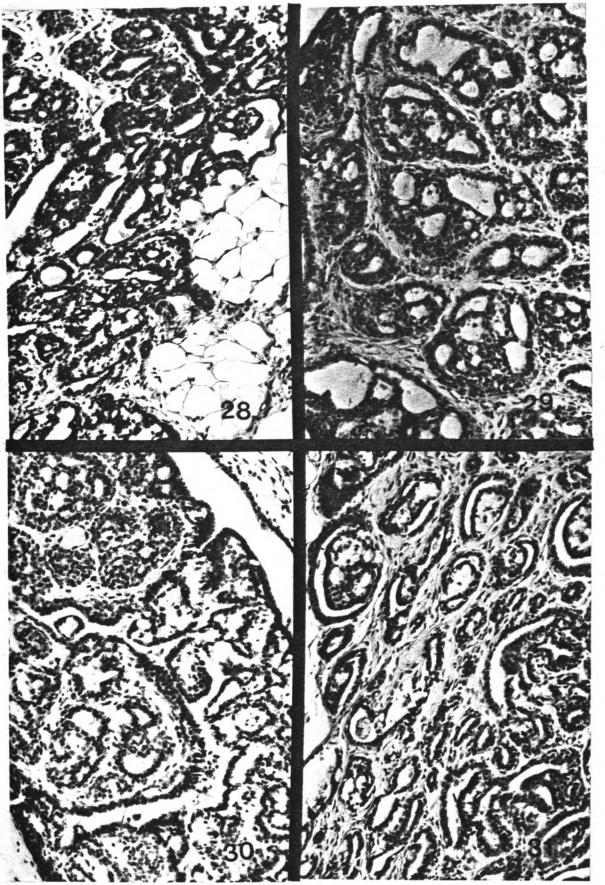
Samples taken from transplants of primary tumor C all showing similar histological pattern to that of parent tumor (Figure 21).

Figure 28 Fragment placed into a gland-containing fat pad. 200x.

Figure 29 Fragment placed into a gland-containing fat pad. 200x

Figure 30 Fragment placed itno a gland-free fat pad. 200x

Figure 31 Fragment placed into a gland-free fat pad. 200x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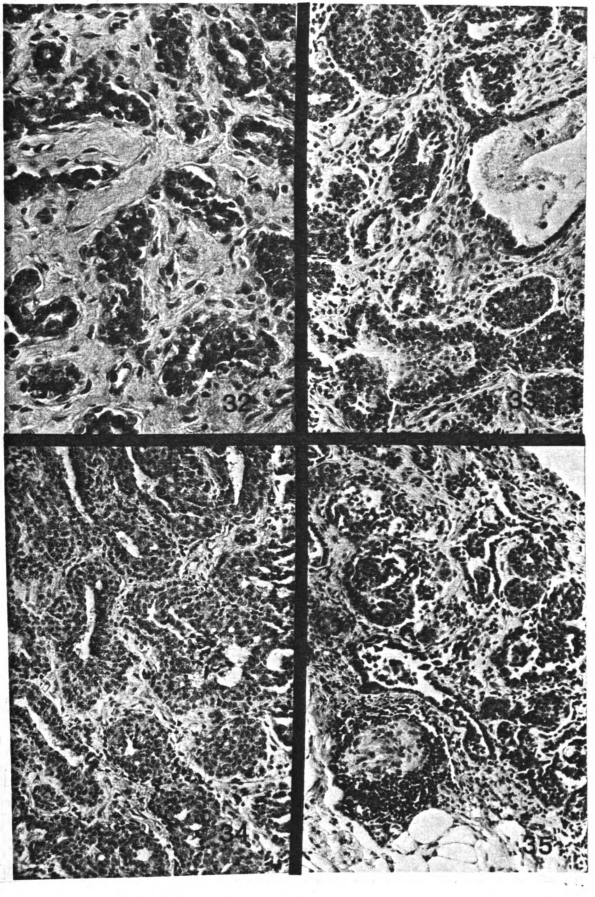
Transplants taken from primary tumor H. Tumors were not palpable prior to the day of termination. Fibrous tissue prevalent.

Figure 32 Fragment placed into a gland-free fat pad. Note the islets of epithelial cells in the stroma. 400x

Figure 33 Enzyme-dissociated transplant placed into a gland-free fat pad. 200x

Figure 34 Enzyme-dissociated transplant placed into a gland-containing fat-pad. 200x

Figure 35 Fragment placed into a gland-free fat pad. Note the ductal and alveolar epithelium within fibrous stroma. 200x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DISCUSSION

The main purpose of this study was to examine differential growth capabilities of primary rat mammary tumor transplants. Enzyme - dissociated tumors were used to determine whether cell suspensions would produce more consistent results than fragments after transplantation. The underlying assumption was that by using cell suspensions, greater control in cell content and number could be achieved. The secondary purpose was to determine how the host microenvironment might affect these differential growth capabilities, since the types of outgrowths have been observed to vary depending on the presence of the mammary parenchyma in the fat pad (Aidells, and Daniel 1974; 1976).

The take frequency included recovery of all local takes, tumors, ductal outgrowths, and hyperplastic alveolar nodule growth. The total recovery of fragments was greater than that of cellular transplants. This is consistent with the statement of Smets (1980), who said that it is an old observation that the implantation of tumor fragments results in higher take frequencies than the injection of tumor-cell suspensions. Enzyme dissociation may interfere with recovery by possibly altering membrane components thereby affecting the capability of the cells to clump, thus making the recovery of the transplants difficult unless outgrowths are present. Peripheral cells are damaged when fragments are excised but the internal cells of the fragment

remain intact and contiguous, which may be a factor in the recovery of fragment transplants over enzyme-dissociated transplants.

There were no concordant findings of inhibition or enhancement of tumor development that was due to enzyme dissociation. The data reflect the individual nature of the primary tumors in support of other findings in the carcinogenesis of individual tumors. In 1970 Prehn, studying methylchoanthrene-induced sarcomas in mice, found that different sublines within individual primary tumors showed marked differences in antigenic specificities and effective antigenic strength. He suggested that such cellular heterogeneity might be characteristic of every primary tumor. Haslam (1979) showed that two classes of primary mammary tumors were found in DMBA-treated Lewis rats. The first was progressively growing tumors most of which were ovarian-dependent (65%), and a smaller proportion, ovarian independent. The second class was designated spontaneously regressing tumors.

Both enzyme-dissociated transplants and fragment transplants gave rise to tumors, ductal dysplasias, and hyperplastic alveolar nodules. Hagar et al. (1978) suggested that the relative mixtures of subpopulations determine the subsequent expression. These investigators found fluctuations in clinical and immunological parameters after having serially transplanted sublines from mouse mammary tumors. Accordingly, subpopulations with similar characteristics might be selected out for expression at any given time. There may be a dominance of one cell type over another due to host selective pressures of variant subpopulations of clones with increasing survivability (Nowell, 1976) or growth behavior of heterogeneous cell populations may be determined by a dominant cell type, e.g.

hormone-independent mouse mammary tumor cells over hormone-dependent cells (Sluyser et al., 1981). Thus, whether the transplant is a random fragment or cellular suspension, it appears to be the content that determines the outcome. Another consideration in accounting for the variablity of outgrowths is that the degree of lability or differentiation as a result of clonal evolution (Nowell, 1976) or that progression of clones following one of alternative paths of development (Foulds, 1975) might determine tumor phenotype or growth potential of the transplants. The greater the stability of the tumor phenotype, the less it is subjected to environmental influence as reviewed by Woodruff (1982). Other evidence was presented by Rivera and Vijayaraghavan (1982) who reported that randomly selected primary mammary tumor transplants often produce ductal outgrowths and/or hyperplastic alveolar nodules in gland-free fat pads. However, ovarian-independent tumor transplants tend to breed true suggesting greater stability of these types of tumors. Therefore, if phenotypically stable tumor cells are considered to be autonomous, they might be expected to produce tumors exclusively, as did samples from tumors D and J (Tables 1, 2 and 7 and from the tumor lines ST-29 and T41-8. More labile tumors might be expected to show a more diverse growth potential giving rise to ductal and hyperplastic outgrowths as well as tumors. Tumors B, F, G, and H provided samples that exhibited this diversity in growth pattern (Tables 1 and 2).

Gland-free fat pads favored ductal and hyperplastic alveolar nodule growth over the fat pads containing mammary parenchyma. It is possible that the potential for tumor cells to undergo differentiation is triggered by the gland-free fat pads (Rivera and Vijayaraghavan,

1982b). To the contrary, there was a suppression of ductal/HAN outgrowth development of fragment and cellular transplants by the gland-containing fat pad. In the intact animal, both fragments and cells had a tendency to remain localized if no tumor formed. This may have been caused by a regulatory or inhibitory effect of normal glands on the transplants, as suggested by Faulkin and DeOme (1960) and Aidells and Daniel (1976). Inhibition was also noted when normal cells were recombined in culture with nodule cells, suggesting a sensitivity of preneoplastic mammary tissue to regulation by factors produced by normal mammary cells (Medina et al., 1978).

These data further show that tumor development was not suppressed by the gland-containing fat pad and are in agreement with other studies demonstrating that highly tumorigenic cell populations override the inhibitory effects of normal glands (Faulkin and DeOme 1960; Aidells and Daniel, 1976). Slemmer (1974) suggested that the expression of the malignant phenotype involved the loss of responsiveness to normal mammary cell regulatory factors. There may be tumor growth factors that allow the tumors to override any regulation or inhibition of their growth. DeLarco and Todaro (1978) Todaro et al. (1979) found polypeptide growth factors produced by tumor cells and virus-transformed cells. In addition, Knauer et al. (1980) found a somatomedin-like polypeptide produced by mouse mammary tumors. Another possibility is that cell types that coexist within the same tumor may provide their own control mechanism, thereby controlling their own development as suggested by Miller et al. (1980).

Not only was there no evidence of autonomous tumor supression, there was no evidence of enhancement of tumor development by the intact

fat pad in all cases. It should be noted that fragment samples from two tumors (B and H) out of nine did grow better in intact than in gland-free fat pads. On the other hand, fragment transplants of tumor J produced tumors regardless of age, parity, or fat-pad condition. Thus, environmental conditions had variable influences depending on the original primary tumor. These findings further support the concept of tumor heterogeneity.

The latency of the tumors was not affected by the condition of the fat pad but was affected by whether or not the tumor was subjected to enzyme dissociation. If enzyme dissociation does alter membrane components, cell-cell interactions that may be necessary for tumor development are also altered, thus affecting the ability of the cells to reaggregate. One exception was tumor H, where the enzyme-dissociated samples had a shorter latency than their fragment counterparts. The shorter latency may have been caused by the presence of a higher proportion of tumor-producing cells in the inoculum than in the fragments, or these particular cells were able to express themselves better without restriction of the connective tissue as suggested by Richards and Nandi (1978), or the inoculum contained clumps making reaggregation time shorter.

Tumor invasion occurs when the main tumor spreads and breaks through the body. In the present study, tumor J was unique in that many of its samples produced invasive tumors. This characteristic was not affected by fat pad conditions, age, or parity but neither was it passed on to the next generation. Indeed the second generation of transplants from tumor J showed the same high tumor growth potential as the parent tumor but was without invasive potential. In other studies,

melanomas and sarcomas contain subpopulations of cells that vary in metastatic or invasive properties (Poste and Fidler, 1980; Fidler and Kripke, 1977; Hart and Fidler, 1981).

Ovarian dependency was a unique characteristic independent of latency, the type of transplant, or the condition of the fat pad. This finding supports the theory that characteristics of tumors progress independently of one another (Foulds, 1975). In another study, independent progression of clinical characteristics of mammary tumors such as latency, growth rate invasiveness, and metastatic capacity, was determined (Hagar et al., 1978). Ovarian dependency was found to be independent of growth rate or types of outgrowths produced from in vitro transformed adenoma cells (Richards and Nandi, 1978).

The tumors varied in responsiveness to ovariectomy. All of the samples from only one tumor of the six showed definitive regression after ovariectomy. Samples from the other five primary tumors were not all responsive to ovarian removal indicating at least two populations of cells within one tumor. The importance of hormones in human breast cancer and the presence of estrogen receptors allow some predictability (55-60%) as to whether these tumors will respond to ovariectomy or antiestrogen drug therapy (McGuire et al., 1975). MacFarlane et al. (1980) showed that this assumption was not always accurate since some tumors with high estrogen receptor content are insensitive to this mode of treatment. Bichel et al. (1982) investigated estrogen receptor content and ploidy and found wide variability within the same tumor as well as among tumors.

Similar hormone studies have been done using DMBA-induced rat mammary tumors. Variability was found in the content of estrogen

receptors such that tumors with high estrogen receptors may be ovarian independent (DeSombre et al., 1976; Boylan, et al., 1975). In addition, prolactin as well as estrogen can regulate tumor growth (Bradley et al., 1976; Meites, 1972; Costlow and McGuire, 1978; Welsch and Meites, 1978). Studies using size regression as an indicator of hormone dependence have shown that ovariectomy will give variable results because prolactin secretion and estrogen interaction must be considered (Bradley et al., 1976). Thus, the variability seen in the tumors from transplants may reflect variability of hormonal dependence of the different subpopulations of the parent tumor.

All of the primary tumors tested were adenocarcinomas by histologic criteria. Although morphology does not give clues as to the clinical nature of adenocarcinomas, there may be some relevance in determining highly differentiated tubules and papillary structures, irregular acini, and highly anaplastic appearance to the maturity of the tumor (Young, et al., 1963; Young and Hallowes, 1973). The morphological variability observed in the tumors from this study was not influenced by host factors or treatment of the transplants.

There were some consistencies in the data worth noting. Tumors with short latencies were associated with high tumor growth potential. There were steady increases in the size with massive internal degeneration as observed by samples from tumors D and J. Which varied in their response to ovariectomy. In some cases there was size regression but in other cases there was apparent growth with complete internal necrosis inside fibrous capsule. According to Young and Cowan (1963), tumors may undergo spontaneous regression independently of their hormonal state. Because some of the tumors examined from

ovariectomized hosts had no apparent living tissue, ovariectomy may have speeded up cell death.

The hormone-dependent mammary tumor line, ST29, showed high tumor growth and was not affected by enzyme dissociation or the condition of the fat pad. The intact fat pad may be necessary initially to support the ovarian dependence but repeated transplantation would tend to decrease variability in such a way that a particular characteristic can be selected for as suggested by Trope (1981). The decreased variability observed in the hormone-dependent line may relate to the phenotypic stability in high tumor-producing primary tumors in that tumors were formed exclusively. On the other hand, the tumor-producing capabilities of the hormone-independent line (T41-8) were affected by enzyme dissociationin that no tumors developed. All fragments produced tumors exclusively with 100% growth potential in both gland-free and gland-containing fat pads. It should be noted that an earlier study done on another hormone-independent line (T-52) did produce tumors from enzyme-dissociated transplants. Therefore, the inability of the cells to form tumors in this instance may be peculiar to this line (T-41).

In conclusion, all data presented support the concept of tumor heterogeneity among different tumors and within the same tumor. It has been shown in morphology, individual growth patterns, differential growth capabilities, and hormone dependency. The same variability was observed in transplants irrespective of the type of transplant used. However, the gland-free fat pad favored ductal/HAN outgrowths while presence of the normal gland suppressed this type of proliferation. Tumor development was not suppressed by the intact gland and was favored in some instances. Thus, as the tumor progressed towards

phenotypic autonomy and stability, the tumor seemed to maintain its own existence with decreasing influence from the host microenvironment. This is in agreement with Faulkin and DeOme, 1960; Aidells and Daniel, 1976; Slemmer, 1974; Rivera and Vijayarhagavan, 1982b; Woodruff, 1982).

In the quest for cancer cures, techniques such as cell cloning and *in vitro* combination with *in vivo* studies are useful tools to manipulate environmental conditions. Specific interactions between normal host mammary epithelium during various reproductive stages and tumor cells can be examined. Studies can be done on tumor growth factors and their effect on the tumor itself as well as their effect on normal mammary epithelial host cells. Finally, age studies on early and late passaged normal mammary epithelium and its *in vitro* transformation can be related to comparable *in vivo* conditions.

In order to understand breast tumors and other dysplasias, tumor cell-cell communication and tumor-host interactions and their effects on phenotypic expression must be studied further such that effective treatment can be administered.

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