Breast Tumor Cell Lines From Pleural Effusions^{1, 2}

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SUMMARY—During 1973, 4 new epithelial tumor cell lines were isolated from pleural effusions from breast cancer patients. We describe 3 of these lines: MDA-MB-134, with a mean chromosome number of 43; MDA-MB-175, with a mean chromosome number of 49; and MDA-MB-231, with a mean chromosome number between 65 and 69. We isolated the same cell type from 4 of 10 effusions from MDA-MB-134 and from 6 of 8 effusions from MDA-MB-175. We found that pleural effusions as a source of breast tumor cells to be cultured and studied *in vitro* have the following advantages: 1) large amounts of material and the possibility of obtaining sequential samples from the same patient; 2) high viability of tumor cells; 3) scarcity or absence of fibroblasts; and 4) the possibility of separating the tumor cells from other "contaminating" cell types by differences in their speed or degree of attachment to the flask. All lines from different patients differed, as seen grossly and microscopically. All lines from sequential pleural effusions from the same patient were apparently alike. No viruses or mycoplasmas were detected in any line.—*J Natl Cancer Inst* 53: 661-674, 1974.

THE ESTABLISHMENT of tumor cell lines directly from solid breast carcinomas has been difficult and frustrating. In 1958 Lasfargues and Ozzello (1) established the first line, BT-20. Dobrynin (2) in 1963 was the next to succeed but, despite many attempts by numerous investigators, no further authentic cultures were reported until 1970. Recently Bassin et al. (3) and Plata et al. (4) isolated lines HBT-3 and HBT-39, and Trempe and Fogh (5) reported on line SK-Br-3. We have been unable to establish lines from more than 200 solid breast carcinoma specimens. Cultures would grow or survive a few days to several months and then were either overrun by fibroblasts or degenerated and died.

Pleural effusions were considered as a good source of breast tumor cells for the following reasons: 1) Generally they provided a plentiful supply of tumor cells; 2) fibroblasts were either rare or absent; 3) the cells in pleural fluid usually showed a viability of 70% or better; and 4) sequential samples from the same patient were sometimes obtained, which allowed replication of studies and the correlation of cell viability or type with the treatment the patient received. This report deals with 3 distinct cell lines isolated from pleural effusions from 3 patients with breast carcinoma.

MATERIALS AND METHODS

Pleural effusions.—These were treated as described by Young et al. (6) and Cailleau et al. (7).

The aseptic and heparinized pleural effusion was transferred into 50-ml plastic tubes, in which it was centrifuged for 10 minutes at 1000 rpm. The clear fluid was decanted and stored after addition of the

antibiotics gentamicin and carbenicillin. (This clear effusion was later filtered and used as an additive at a concentration of 7–10% to some of our basic media.) If the precipitated cell pellet contained much blood, it was resuspended and mixed in 10 volumes of distilled H₂O. This suspension was then rapidly brought to isotonicity with 1 volume 10× Eagle's minimum essential medium or Waymouth's MB752/1 medium, and a few drops of sterile 3% NaHCO₃ were added to bring the pH to 7.4. This suspension was recentrifuged and decanted; the pellet was resuspended in growth medium. Small portions were set aside for electron microscope (EM) studies and Papanicolaou staining. The remaining cells were tested for viability with the use of trypan blue and distributed into both plastic and glass flasks containing various media. These tests have shown that the tumor cells retain essentially the same viability as before treatment with distilled water, providing the return to isotonicity is made within 1–2 minutes. Growth of the cells in culture also confirms this. If sufficient cells were present, portions were placed in ampules with either 10% glycerin or 10% dimethyl sulfoxide for freezing and storing in liquid nitrogen.

Media.—Our basic media consisted of BTN or CIG⁵ [Cailleau et al. (7)] and a recently modified and simplified medium, CIGC, consisting of 80% Leibovitz' L-15, 20% fetal bovine serum (all prepared media and serum were obtained from Grand Island Biological Co., Santa Clara, Calif.), 16 µg glutathione/ml (Calbiochem, Dallas, Tex.), 10 µg insulin/ml (zinc-crystalline, Elanco Products Co., Indianapolis, Ind.), and antibiotics in the form of 100 µg disodium carbenicillin/ml (Pyopen, Beecham-Massengill, Dallas, Tex.) and 50 µg gentamicin/ml (gentamicin reagent solution, Schering Diagnostics, Port Reading, N.J.).

MDA-MB-134.—This cell line was isolated from 4 of 10 specimens of pleural effusions from a 47-year-old white woman who had had a right radical mastectomy in May 1970 for an infiltrating duct carcinoma with axillary node metastasis. She developed a left axillary node metastasis in July 1971 and a right

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⁵ CIG is a modification of L-15 (containing cortisol, insulin, and glutathione) which Colonel Leibovitz had devised and initially provided for our studies.

pleural metastasis with effusion in July 1972. At oophorectomy (Aug. 1972) her abdominal lymph nodes were enlarged; peritoneal washings contained adenocarcinoma cells, presumably of breast origin. Oophorectomy did not decrease effusion formation, and thoracenteses of about 1.0–1.6 liters were required every 4–6 weeks. The pleural fluid always contained viable tumor cells. Despite her illness, the patient remained active and nearly free of symptoms except for increasing dyspnea just before each thoracentesis. The rate of effusion reaccumulation increased in July and August 1973. Systemic 5-fluorouracil (5-FU) was started August 29, 1973. A thoracentesis on September 25, 1973, yielded 750 ml of sanguineous fluid with cell viability greatly reduced. The effusion has not recurred, and the patient has been clinically well.

MDA-MB-175.—This cell line was obtained from 6 of 8 pleural effusions drawn biweekly from a 56-year-old black woman, who had had a left simple mastectomy on December 1, 1972, for an infiltrating duct mammary carcinoma. When first seen at this hospital on January 10, 1973, this patient had clinically involved axillary nodes and a pleural effusion. Thoracenteses were done repeatedly; each produced about 1 liter of bloody effusion containing viable malignant cells. Initially the patient was comfortable and active, with effusion and small nodal metastases the only evidence of tumor activity. Her condition later deteriorated, and systemic therapy with cyclophosphamide and prednisone was started on May 30, 1973. The rate of pleural fluid reaccumulation decreased thereafter; cell viability was low in the single specimen of July 10, 1973, collected 19 days after a second cyclophosphamide and prednisone treatment. The patient died on July 22, 1973. No autopsy was done.

MDA-MB-231.—This cell line was established from a single sample of pleural effusion obtained October 17, 1973, from a 51-year-old white woman, who had had a right radical mastectomy in January 1969 for a poorly differentiated tumor tending toward papillary configuration and tubule formation. This patient also had an intraductal carcinoma. A pericardial effusion and a left pleural effusion appeared in June and July 1973, respectively. Both contained malignant cells compatible with a primary tumor of the breast. Oophorectomy (July 3, 1973) and subsequent systemic treatment with 5-FU and prednisone were ineffective. In September 1973, combined chemotherapy (cyclophosphamide, adriamycin, and amethopterin) was initiated, without significant benefit. As of December 21, 1973, the patient was bedridden and required thoracenteses at weekly intervals. The patient died January 13, 1974.

RESULTS

Line MDA-MB-134

Ten sequential pleural effusions were obtained; results were relatively consistent (table 1). When the cells in the fresh pleural effusion showed a viability

of 70% or higher, they grew well in tissue culture. Several cell types were present in all samples. Only a few clumps of epithelial cells adhered to the flask, along with a preponderance of mesothelial-like or inflammatory cells. The cells we eventually isolated as a continuous line initially did not stick on the surface of the flasks and were usually discarded when media were changed. These cells were observed in all pleural effusions from this patient but were not concentrated and transferred successfully until our sixth to ninth samples. These small, round or short, spindle-shaped cells floated in the fluid as small clumps or grapelike clusters and either remained suspended or detached easily from the surface of the flask by being shaken. After several weeks in culture and successive transplants of the detached cells, pure cultures of this small cell were established (fig. 1). Later cultures attached to the glass surface more readily but could be loosened by being vigorously shaken or gently scraped. These cells were studied by EM (figs. 5–7) and histologic staining techniques; they appeared to be epithelial tumor cells. Chromosome counts were hypodiploid, with most chromosomes ranging between 41 and 45 with a mean of 43 (fig. 10).

The chromosomal configurations of 2 sublines established from the sixth and eighth pleural effusions were examined. The karyotypes were essentially identical: a mean of 43 chromosomes with the same extra-long markers in most cells.

Because the size, shape, and detachability of these cells differed from those of our other established lines, the possibility of their being of lymphoid origin was considered. However, both our hematologist and cytologist diagnosed these cells as being probably of tumor and nonlymphoid origin. The histologic sections (not done in our hospital) made from the original breast tumor were of poor quality. Nevertheless, pathologic examination revealed the breast tumor to be composed of relatively small cells. This also agrees with our findings. Our final criterion for considering these cells to be of breast tumor origin depended on the EM pictures which were compatible with those of tumor cells.

Once it was realized that the small floating cells might be tumor cells, we could culture them from 4 of the last 5 sequential pleural effusions. This cell type seemed identical in all pleural effusions, and chromosome-banding studies are in progress to verify this hypothesis. Viability of the cells in the pleural effusions was 50% or higher in all but the last sample. This sample was taken 19 days after treatment with 5-FU, showed a lower viable cell count (40%), yielded only a few tumor cells, and did not give rise to a cell line.

Line MDA-MB-175

Eight sequential pleural effusions were obtained over a 4-month period and yielded 6 tumor cell lines ostensibly indistinguishable from each other (table 2).

The 2 pleural effusion samples not yielding a cell line were different from the rest. Sample 5 was allowed to stand 3 days at room temperature before being

TABLE 1.—Cells found in sequential pleural effusions from patient MDA-MB-134

Sample No.	Date	Volume (ml)	Cell viability (%)	Cell types*	Culture survival (months)	Established cell line
1	10/23/72	1500	>95	E,R M,F,D	3-4 4-5	—
2	12/12/72	1050	75	E,R M,F,D	E,1: R,7 5-7	—
3	1/11/73	1000	95	E,R M,F,D	1-2 2-3	—
4	2/12/73	800	70	E,R M,F,D	1-2 2-3	—
5	3/27/73	900	Not done	E,R M,F,D	3-4 3-4	—
6	5/ 1/73	1100	50	E,R M,D	E,2 2-3	R
7†	6/12/73	1200	50	E,R M,F,D	E,2 2-3	R
8	7/24/73	1700	70	E,R M,F	E,2-5 2-3	R
9	8/28/73	1750	85	E,R M,F,D	E,4-5 2-3	R
10‡	9/25/73	750	40	E,R M,F,D	1-2 4-5	—

*E=epithelial (presumably tumor) cells; R=small, round (epithelial-like) cells established in 4 cultures from 4 pleural effusions; M=mesothelial-like cells; F=fibroblast-like cells; and D=dendritic (presumably inflammatory) cells.

†Left 72 hr at room temperature before being cultured.

‡Pleural fluid drawn 19 days after 5-FU treatment.

TABLE 2.—Cells found in sequential pleural effusions from patient MDA-MB-175

Sample No.	Date	Volume (ml)	Cell viability (%)	Cell types*	Culture survival (months)	Established cell line
1	3/ 6/73	1300	90	E M,D,F	∞ † 3-4	E
2	3/19/73	1000	90	E M	∞ 2-3	E
3	4/ 2/73	1000	40	E M,D,F	∞ 1-2	E
4	4/17/73	1000	75	E M,F	∞ 3-4	E
5	5/ 1/73	1000	5	—	No cell growth	—
6	5/16/73	900	90	E M,D,F	∞ 3-4	E
7	5/30/73	700	Not done	E D,F	∞ 2	E
8‡	7/10/73	330	50	E M,F	2 wk 1	—

*E=epithelial (presumably tumor) cells; M=mesothelial-like cells; D=dendritic (presumably inflammatory) cells; and F=fibroblast-like cells.

†∞=considered to be an established cell line, capable of multiplying indefinitely.

‡Pleural fluid drawn 19 days after cyclophosphamide and prednisone treatment.

processed. Only 5% of the cells were viable, and no cell growth was obtained. The final sample (#8) was taken 19 days after chemotherapy treatment with cyclophosphamide and prednisone. The viability of these cells was 50%, and the few cells present survived only 2 weeks.

These cell lines appeared entirely different from line MDA-MB-134. Many tumor cells were present, and the extraneous cell types were fewer (table 2). Cells which formed tight-spreading or domed clumps appeared as the major or only surviving cell type. These cells continued to grow in tight colonies and to multiply slowly (fig. 2). During the first few months, cultures were divided 1:2 about once a month and, at present, are transferred at 2- to 3-week intervals at dilutions of 1:2 to 1:5. Electron micrographs (fig. 8)

and histologic and cytologic studies all point to their epithelial and tumor origin. These cell lines have a mean of 49 chromosomes with several characteristic markers (fig. 11).

Results similar to those obtained with line MDA-MB-134 were found when we karyotyped 2 sublines (III and IV) of this culture. The mean chromosome number remained 49 or 50, and the characteristic markers also were present in the great majority of the cells.

Line MDA-MB-231

A third tumor line was recently isolated from a single pleural effusion. The sample was bloody but contained many tumor cells which showed a viability of 40%. The patient from whom this sample was

obtained had had varied treatments and had received combined chemotherapy (cyclophosphamide, adriamycin, and amethopterin) 3 weeks before this fluid was drawn. Several flasks with different media were inoculated with the washed and concentrated cells. A preponderance of mesothelial-like and some dendritic-like cells were seen after a few weeks in culture. In only 2 flasks did typical epithelial-like clumps or colonies appear. These cells then spread out, overgrew most surrounding cells, and formed clumps of round or spindle-shaped, granular cells. As they piled up, balls or clumps of these cells could be shaken off into the liquid and transferred to another flask free from the other "contaminating" cell types. Within a few weeks, line MDA-MB-231 became established as a pure epithelial cell line. This line grew rapidly and, at dilutions of 1:10 or higher, had to be transferred every 5–8 days. The cells remain granular and either form a loose network of round or short, spindle-shaped cells or pile up as heavy clumps of round cells which can be shaken off the glass (fig. 3). Typical EM and chromosomal studies are shown in figures 9 and 12. This line has a near-triploid chromosome number, mainly ranging from 60–70, and contains several characteristic markers.

When these 3 lines are compared with our line MDA-MB-157 (fig. 4), they are morphologically distinct and have quite different growth patterns. Chromosome configuration and chromosome numbers are also characteristic of each cell line.

As of May 16, 1974, the lines have been in continuous culture as follows:

1) MDA-MB-157, isolated 1/2/73, has been subcultured between 45 and 50 times at dilutions of 1:5–1:20 every 5–10 days since 4/1/73. Growth of this line in an antilymphocyte serum-treated BALB/c mouse is illustrated in figure 13.

2) MDA-MB-134 has 4 sublines established 5/1/73, 6/12/73, 7/24/73, and 8/28/73. The lines are now in subcultures 15–20. They are usually diluted 1:2–1:5 by being shaken or scraped and are transferred every 2–3 weeks with changes in media every 4–7 days.

3) MDA-MB-231 has been in culture since 10/17/73 but is considered an established cell line because of its uniform and rapid growth. It has been transferred at least 40–50 times at dilutions of 1:5–1:50. At present, it becomes confluent in the flask about 4–5 days after transfer at dilutions of 1:10–1:40. Although this line has been in culture only 7 months, it grows rapidly and vigorously and has produced tumor nodules in 6 weeks when injected into nude mice (fig. 14).

4) MDA-MB-175 has 6 sublines (I–IV, VI, VII) isolated between 3/6/73 and 5/30/73. The cells grow in tight clumps and multiply slowly. Until recently, they were subcultured at 1:2 or 1:3 dilutions about once a month. At present, they can be subcultured at 2- to 3-week intervals and are in passages 11–18.

DISCUSSION

Since January 1973, we have isolated 4 breast tumor lines from pleural effusions. In our first line (MDA-MB-157), the pleural effusion had stood over 72 hours at room temperature and we still succeeded in culturing the cells (6). In 2 later lines, sequential effusions from the same patients yielded 6 of 8 and 4 of 10 sublines, respectively, each of which resembled the original line.

We believe that pleural effusions are an excellent "milieu" for preserving the tumor cells. The filtered, cell-free effusions are used in our own milieu, being incorporated at a concentration of 7–10% in 1 or more of our media. Generally these effusions seem to prove beneficial for the initial culture of the tumor cells. Once the cells become established cultures (after 3–6 months or more), simpler media can be substituted.

Our sequential samples of MDA-MB-134 showed varying degrees of success. We might have established a cell line sooner if we had been aware that the small, floating cells were not lymphocytes and should not have been discarded with the used media. Once we concentrated and transferred these floating cells, they continued to multiply. Sample 7 has yielded only 1 viable culture from 12 or more initial cultures and has grown slowly. This may well be due to the 24-hour delay before culturing, which lowered the viability of the total cell population to 50%. Sample 10 never really showed enough viable tumor cells in culture to allow them to get a foothold. Again the viability was low (40%), and the drug treatment was apparently effective, as the patient has been in remission since that time.

Good cultures of tumor cells were easily obtained from all pleural effusions from patient MDA-MB-175 except samples 5 and 8. In sample 5, only 5% of the cells were viable after 72 hours at room temperature; since the effusion was bloody and hemolyzed, nothing grew. In sample 8, drug therapy apparently reduced the viability of those cells in the effusion to around 50% and, while some cells of all types attached to the flasks, they did not survive more than 1 month.

In establishing these tumor lines, we found that 3 or more cell types were usually present in the pleural effusions. However, we could obtain at least partial separation of the tumor cells by using the difference in the length of time it took them to attach to the flask. Frequently the tumor cells tended to be less "sticky" than the other cells; therefore, we could separate these tumor cells from the other cells by pouring or pipetting the supernatant fluid and its floating balls or clusters of tumor cells into a new flask. We could do this several times, starting 4–24 hours after the initial inoculum. Mesothelial-like cells were rare during the first 2–3 weeks of culture, then grew rapidly and generally degenerated and died in 3–4 months. Dendritic-like cells, numerous in the first few weeks of culture, did not seem to multiply and survived only 2–3 months. Both these cell types adhered tightly to the surface of the flask and could not be shaken off.

In the 2 established cell lines in which we did not receive sequential samples, the tumor cells were extremely hardy. MDA-MB-157 cells were able to proliferate, even though the pleural effusion had stood for 4 days at room temperature (6). MDA-MB-231 cells showed only a 40% viability in the original pleural effusion but established themselves in culture, despite the adverse effects of massive chemotherapeutic treatment of the patient. In MDA-MB-157, MDA-MB-175, and MDA-MB-231 the dominant tumor cell, after a lag period of 6 weeks or more, began to outgrow the other cell types, which were then beginning to degenerate. Once the colonies or plaques of tumor cells were visible through the flasks, they were easily established in culture. Where the tumor colonies were tightly adhering epithelial colonies, they could either be picked off and transferred to new flasks or the surrounding mesothelial-like cells could be scraped away to allow the tumor cells to spread out on the surface of the flask. Where the cells formed clumps or domed colonies, these could be shaken off and thus freed from the nontumor cells adhering tightly to the flask. By gently shaking or simply pouring off the used culture media, we could easily separate MDA-MB-134 from all other cells in the original culture.

It may not be too surprising that, in several sequential samples from 2 different patients, we have been able to establish cell lines and that, in each instance, the persisting cells were microscopically identical. In each of our 4 lines, 3 of which are described here, the established line has a characteristic appearance: different rate of growth and chromosome number. Chromosome-banding patterns and karyotypes of the 4 sublines of MDA-MB-134 and the 6 sublines of MDA-MB-175 are being studied, but preliminary results seem to show remarkable consistency.

Apparently the malignant cells surviving in the patient after mastectomy and radiation or chemotherapy and accumulating in the pleural effusion are sufficiently hardy to adapt *in vitro* in about 10% of the pleural effusions we studied. Those pleural effusions yielding positive cultures and established cell lines did so repeatedly when sequential samples were available under similar conditions.

Other workers who have isolated cell lines from pleural effusions have not had sequential fluids available. The lines, Lev II, Lev III, and Sal III isolated by Beth and Olds in 1967, are mentioned by Giraldo et al. (8) but no pertinent data have been published; the cells are now considered to be of lymphoid origin. Soule et al. (9) noted the same types of accompanying or contaminating cells with line MCF-7 and the ability of the tumor cells to remain "free-floating" and thus be purified by mechanical separation. Seman (10) also found the same behavior in isolating his cell line.

It may be considered more accurate to obtain the breast tumor cells from breast tumor tissue at mastectomy, but the percentage of cell lines so established has been small. Many factors intervene: the lower

percentage of viability of the tumor cells in most solid tumor samples; the high percentage of fibrous, fatty, and lymphocytic tissue; and the difficulty in separation of the tumor cells from those accompanying cells when attempts are made to culture them.

The fact that the tumors have metastasized and adapted themselves sufficiently to propagate in pleural effusions may indicate a selection of the more active, hardy, or mutated form. The growth in pleural effusion is already an adaptation to a liquid environment and may predispose the cells to grow *in vitro*, especially if the pleural effusion itself is retained as part of the culture media. It is possible to obtain as much as 1–2 ml of highly viable, packed tumor cells, already dispersed in small clumps, relatively free from stroma or easily separated from other cell types. The amount of pleural effusion obtained averages about 1000 ml/thoracentesis, and sequential samples may sometimes be obtained. This allows for a wide variety of experiments, as well as storage in liquid nitrogen for future reference. With proper clinical, radiologic, histochemical, EM, culture, and other techniques, the identification of the cultured cells as being of breast tumor origin should not be in question, any more than those cell lines "presumably" isolated from the solid tissue from the breast. All criteria we have used to date indicate that our lines are epithelial tumor cells, do not contain any viruses or mycoplasmas, and are of breast tumor origin.

Further studies with the use of autoradiographic and immunologic techniques, as well as injections into nude mice, are being done to provide additional evidence of the origin of the cell lines; these will be reported.

To date, injections into antilymphocyte serum-treated and nude mice have yielded tumors from 2 of our 4 lines (MDA-MB-157 and MDA-MB-231). The fixed sections of these tumors (figs. 13, 14) are compatible with human adenocarcinoma and further support our contention that these cell lines are of human breast tumor origin.

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FIGURE 1.—MDA-MB-134. Small, round or short, spindle-shaped cells loosely adhering to glass. Phase contrast. $\times 200$

FIGURE 2.—MDA-MB-175. Compact colonies of epithelial-like cells. Phase contrast. $\times 170$

FIGURE 3.—MDA-MB-231. Granular, round or spindle-shaped cells, tending to pile up. Phase contrast. $\times 200$

FIGURE 4.—MDA-MB-157. Granular, epithelial-like cells with "scalloped" edges. Phase contrast. $\times 200$

Figure 5.—MDA-MB-134. Electron micrograph showing a group of closely apposed cells with moderately high nuclear-cytoplasmic ratio and prominent nucleoli. $\times 7000$

FIGURES 6, 7.—Cells from same culture, showing desmosomes uniting adjacent cells. Figure 6: $\times 36,000$. Figure 7: $\times 33,000$

FIGURE 8.—MDA-MB-175. Group of cells showing intimate apposition of adjacent cell membranes, with frequent desmosomes and rudimentary acinar formation. Numerous microvilli project from apical cell borders. $\times 19,000$

FIGURE 9.—MDA-MB-231. Adjacent cells are closely apposed but desmosomes are relatively few and inconspicuous. Sites of desmosomes are indicated by *arrows*. Note high nuclear-cytoplasmic ratio. $\times 8500$

FIGURE 10.—MDA-MB-134. Shows 43 chromosomes and karyotype. Three markers include 2 extra-long chromosomes (m). Giemsa.
 × 1400

FIGURE 11.—MDA-MB-175. Shows 50 chromosomes and karyotype. Among unidentified chromosomes are 2 extra-long and 1 minute marked m. Giemsa. $\times 1800$

FIGURE 12.—MDA-MB-231. Shows 67 chromosomes and karyotype. Many unidentified markers. Giemsa. $\times 1400$

FIGURE 13.—Tumor obtained after 6 weeks following injection of cultured cells, MDA-MB-157, into antilymphocyte serum-treated mouse. Cells are morphologically similar to those of the patient's primary breast carcinoma. Hematoxylin and eosin (H & E). $\times 550$

FIGURE 14.—Tumor growth in nude mouse 6 weeks after injection of cultured cells, MDA-MB-231. Cells form gelatinous mass; adenocarcinoma cells are in a lymphatic capillary. H & E. $\times 320$