Isolation of a Continuous Epithelioid Cell Line, HBT-3, from a Human Breast Carcinoma (36850)

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(Introduced by S. Baron)

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Interest in human breast carcinoma, as a possible model for virus-induced neoplasia, has been stimulated by several recent reports of possible virus-associated activities in human breast tumor material. Virus-like particles, similar in morphology to the mouse mammary tumor virus and to the virus recently observed in a rhesus monkey breast carcinoma (1), have been observed in human milk (2, 3). In addition, a DNA polymerase possibly associated with RNA tumor virus particles (4, 5) has been demonstrated in human milk samples (6), as has 65–70s RNA (7). Recently, the presence in human breast tumors of RNA related to that of mouse mammary tumor virus has been proposed on the basis of molecular hybridization experiments (8).

Many of the studies with human breast carcinoma have been carried out using tumor material derived directly from *in vivo* sources, a result, in part, of the paucity of well-characterized, rapidly growing human breast tumor cell lines. Human breast tumor cells grow with difficulty under ordinary culture conditions, and reports describing continuous cell lines obtained from primary breast tumor material are rare. Two such lines, one established by Lasfargues and Ozzello (9) from a duct cell carcinoma, and a second derived by Martorelli, Parshley and Moore (10) from a scirrhous carcinoma, have been reported. In addition, a few other cell lines have been established following cultivation of cells from metastatic tumors and pleural effusions obtained from patients with breast tumors. The need for additional cell culture material with which the virology, immunology, physiology and biochemistry of human breast carcinoma can be studied is apparent.

We have investigated the effects of collagenase treatment, which has been shown to facilitate cultivation of epithelial tissue (11), in conjunction with the cloning of cells from tumor explants to prevent overgrowth with fibroblasts, on the growth of human breast tumor tissue *in vitro*. In this paper, we report the isolation of a continuous tumor cell line with a rapid rate of growth and an unusually high cloning efficiency following cultivation of a human breast adenocarcinoma.

Materials and Methods. Cell line HBT-3 was derived from a breast carcinoma occurring in a 50-yr-old woman with a history of three normal pregnancies. Histological examination showed this tumor to be a mucus producing adenocarcinoma, a relatively uncommon form of breast cancer (Figs. 1 and 2). No lymph node involvement was detected after histological examination of several axillary nodes.

Tumor tissue was received in the laboratory approximately 24 hr after surgery. Fatty tissue was separated from the firmer part of the tumor material, which was then rinsed with Earle's balanced salt solution, minced with scissors and scalpels, suspended in 10 ml of 1 mg/ml collagenase (in Hanks' balanced salt solution), and incubated at room temperature overnight. Digested tumor material was then centrifuged at 800 rpm for 30 min, and the resulting pellet was suspended in McCoy's 5a medium containing 15% fetal calf serum and plated in a 75 cm^2 plastic flask (Falcon Plastics, Oxnard, CA). After incubation for 7 days at 37°, the culture was examined carefully for the presence of colonies of epithelioid cells, and a single colony

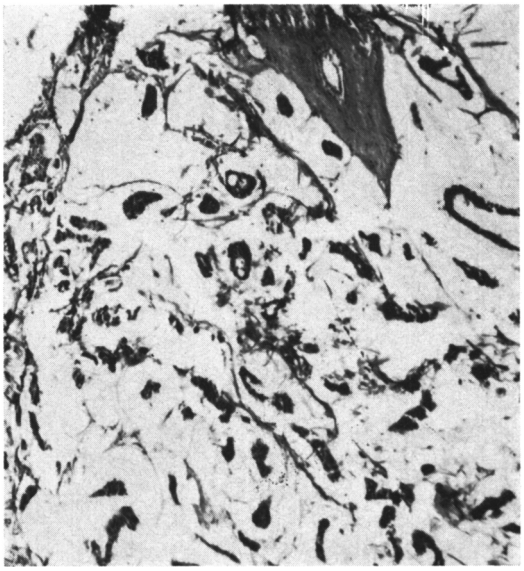

FIG. 1. Low power view of the original tumor from which cell line HBT-3 was derived showing irregular acinar and tubular epithelial structures scattered through prominent mucus lakes. 48×.

was picked from the flask using a Pasteur pipette. The cells from this colony, contained in approximately 1 ml of medium, were plated in microplates (Falcon Plastics, Microtest II plates, capacity 0.2 ml/well) together with fresh medium. After 3 wk additional incubation, colonies of epithelioid cells were observed in 3 of the 12 wells seeded. These cells were removed and seeded in flasks. The resulting cell line, designated as HBT-3, has been in continuous culture since that time, covering a period of approximately 10 mo and over 50 passages at cell dilutions ranging from 1:10 to 1:50.

Media, sera and other tissue culture reagents were obtained from Grand Island Biological Co., Grand Island, NY. Collagenase was supplied by Worthington Biochemical Corporation (Freehold, NJ) as a crude preparation with an activity of 197 units/mg.

For cell doubling time experiments, 50,000 HBT-3 cells were plated in 75 cm^2 Falcon flasks. At 48, 72, and 96 hr after plating, duplicate flasks were examined microscopically. The total number of cells per flask was calculated after determining the average number of cells in each of 50 randomly placed fields.

For chromosomal analysis, HBT-3 cultures from several different passage levels and a fibroblastic culture also derived from the same breast tumor tissues (see below) were employed. Colcemid (0.5 μg/ml of culture fluid) was added to the cultures, which had been fluid changed 24 hr previously. After colcemid treatment for 2–3 hr the culture medium was removed, and 8 ml of a hypotonic solution (0.5%) of KCl was added. The samples were incubated for an additional 30 min, after which a portion of the cells was detached from the monolayers following vigorous shaking of the culture flasks. These cells were lightly centrifuged and fixed in 3:1 methanol–acetic acid. After 3 washes in fresh fixative the cells were dropped on wet slides, stained with Giemsa, and photographed.

Results. Characteristics of human breast carcinoma cell line HBT-3. Following the establishment in culture of cells from human breast tumor HBT-3 (see above), a variety of procedures was employed in an effort to characterize the resulting cell line. HBT-3 cells are epithelioid in appearance (Fig. 3), and when plated very sparsely the cells are capable of forming multilayered colonies

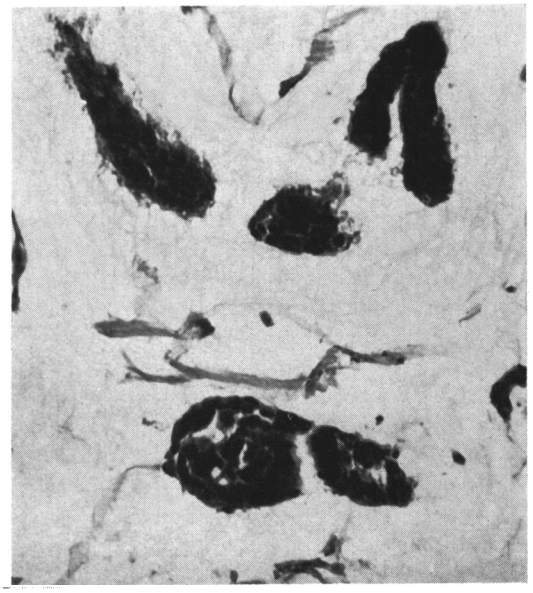

FIG. 2. High power view of the tumor showing epithelioid elements with nuclei exhibiting moderate pleomorphism and hyperchromatism. Note the general resemblance to breast ductal epithelium. 240×.

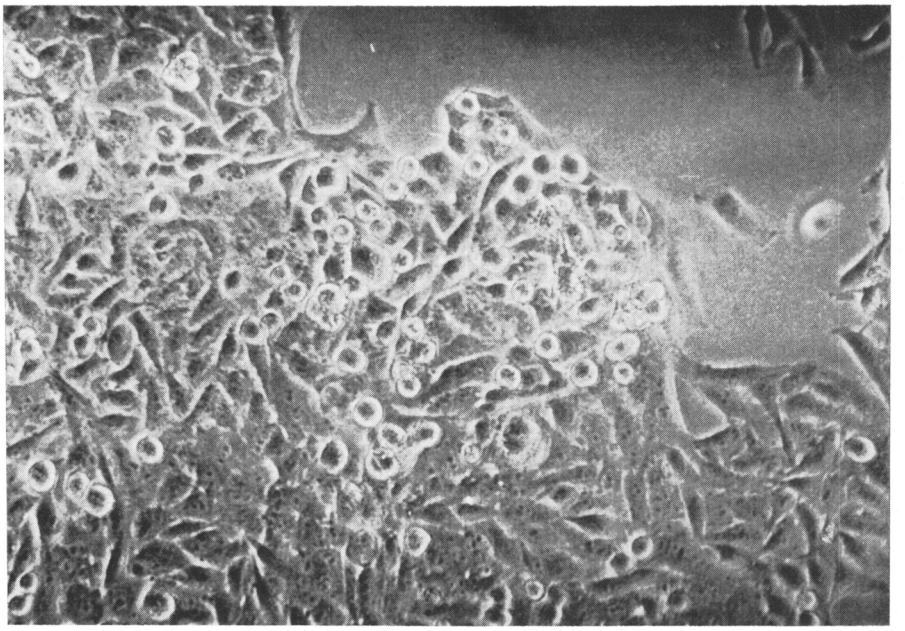

FIG. 3. Actively growing culture of HBT-3 breast tumor cells. Phase contrast. 160×.

(Fig. 4), indicating a lack of contact inhibition of cell growth. While HBT-3 cells can be maintained successfully in several different culture media, they seem to grow best in RPMI 1640 (Grand Island Biological Co.) supplemented with 15–20% heat-inactivated fetal calf serum and 1 ml/100 ml of 200 m*M* *l*-glutamine. Under these conditions HBT-3 cells grow quite rapidly and can be subcultured at a 1:20 dilution approximately every 5 days. Cell doubling time for this line is approximately 18 hr.

The original HBT-3 tumor was also cultured by methods similar to the procedures described above, but without the use of collagenase. Under these conditions, no epithelioid cells were seen, but fibroblastic cells could be recovered (Fig. 5). These fibroblastic cells grew comparatively slowly, showed no evidence of loss of contact inhibition, and could not be cultured for more than 10 to 12 passages.

Immunofluorescent procedures using species specific typing sera (12) as well as lactic acid dehydrogenase isoenzyme patterns both showed HBT-3 cells to be of human origin.

Starch gel electrophoresis (TEB buffer, pH 8.6), performed through the courtesy of Dr. P. R. McCurdy (Georgetown Medical Division, D. C. General Hospital, Washington), indicated that HBT-3 cells contain glucose-6-phosphate dehydrogenase of type A variant mobility. Peripheral leukocytes and hemolysate obtained from the original patient are heterozygous for this enzyme, containing variants of both A and B mobility.

The HBT-3 cell line exhibits many of the properties usually associated with continuous tumor cell lines. HBT-3 cells multiply rapidly, can be cultured for prolonged periods without any apparent decrease in their growth rate, and can multiply well even when plated at extremely low cell densities. The cloning efficiency of HBT-3 cells was estimated by plating single cells from each of several different passage levels in individual wells using Falcon Microtest II plates with a capacity of 0.2 ml/well. Seven to 10 days after plating, wells were scored for the presence of colonies of HBT-3 cells. Under these conditions 70% of the cells were able to give rise to viable colonies. Colonies could be readily picked and grown in mass cultures as clonal lines of HBT-3 cells.

In separate experiments, a high cloning efficiency was observed following plating of the HBT-3 breast tumor line in 75 cm^2 flasks. Two hundred cells per flask were

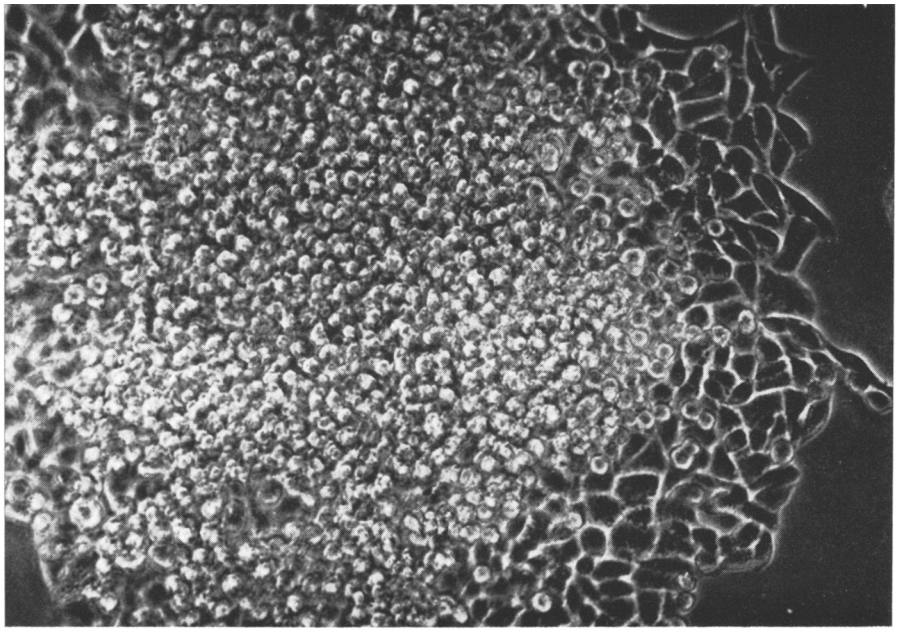

FIG. 4. Multilayered colony of HBT-3 cells arising from a single cell, 9 days after plating. Phase contrast. 160×.

plated and left undisturbed for 9 days, at which time the cells were stained (May-Grunwald), and the colonies were counted macroscopically. An average of 68% of the cells plated formed visible colonies in each of several replicate experiments.

HBT-3 cells were negative when stained with either mucicarmine or periodic acid Schiff, indicating a lack of mucin production.

Karyology of HBT-3 cells. HBT-3 cells from 3 passages, the 23rd, 27th and 43rd, representing cells which had been in culture for 21, 25, and 45 wk, respectively, were selected for chromosomal study. One hundred

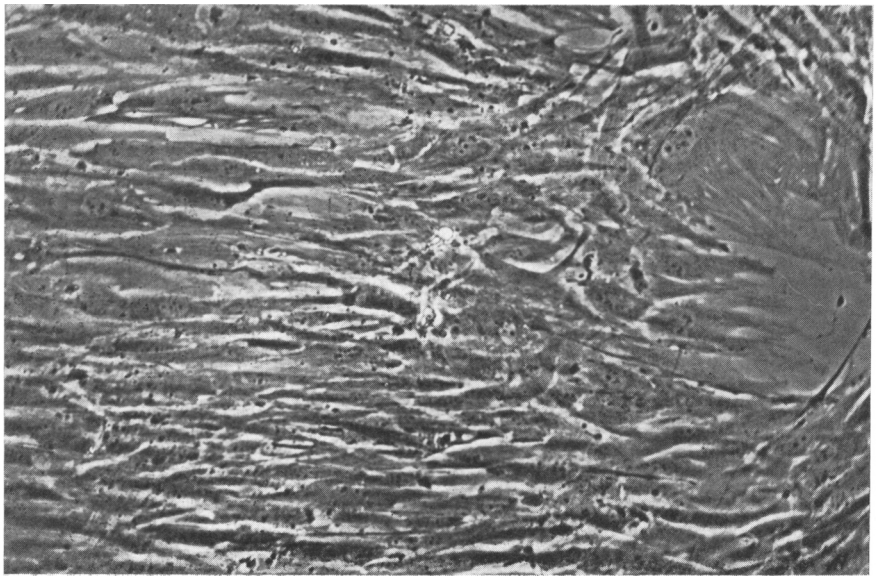

FIG. 5. Fibroblastic cells from breast tumor HBT-3. Phase contrast. 160×.

TABLE I. Chromosomal Analysis of HBT-3 Cells.

A. Passage 23 (21 wk in culture)

No. chromosomes:	62	64	65	66	67	68	69	74	124	136	Total
Frequency	5	10	*20*	*30*	*20*	8	4	1	1	1	100

Frequency (%) of marker[a] chromosomes		Other abnormal chromosomes (%)	
Cells with 1 marker	16	Minutes	6
Cells with 2 markers	22	Dicentrics	4
Cells with 3 markers	62	Rings	4
Total cells with markers	100		

B. Passage 27 (25 wk in culture)

No. chromosomes:	52	55	60	64	65	66	67	68	69	70	72	134	Total
Frequency	2	1	2	3	5	6	5	*27*	*39*	6	3	1	100

Frequency (%) of marker chromosomes		Other abnormal chromosomes (%)	
Cells with 1 marker	30	Minutes	2
Cells with 2 markers	36	Dicentrics	6
Cells with 3 markers	9	Rings	2
Total cells with markers	75		

C. Passage 43 (45 wk in culture)

No. chromosomes:	53	60	66	67	68	69	70	71	76	80	Total
Frequency	2	4	5	9	10	*34*	*28*	5	2	1	100

Frequency (%) of marker chromosomes		Other abnormal chromosomes (%)	
Cells with 1 marker	20	Minutes	2
Cells with 2 markers	24	Dicentrics	6
Cells with 3 markers	0	Rings	0
Total cells with markers	44		

[a] See Figs. 6 and 7.

cells with discrete metaphase plates from each passage were examined. The results are summarized in Table I. The modal chromosome number of HBT-3 cells varied from 66 to 69, shifting slightly in the direction of a higher modal number with continued passage (Table I).

Three different markers were noted (Figs. 6 and 7): (a) A large submetacentric chromosome; (b) a medium-sized submetacentric chromosome with a secondary constriction below the centromeric region; and (c) a smaller submetacentric chromosome with a similar secondary constriction. These markers were seen either singly or in various combinations. All cells of passage 23 showed at least one marker, and 62% showed all 3 markers. As has been noted with other human tumor cell lines, the proportion of marker-containing cells decreased with continued passage in culture (Table I). Other abnormal chromosomes such as dicentrics, rings, minutes and breaks were noted in a relatively small porportion of the HBT-3 cells examined.

Fibroblastic cultures derived from the same tumor tissues which gave rise to cell line HBT-3 (see above) were also processed for karyotypic analysis. The karyotype was that of a normal human female with a modal chromosome number of 46 and no markers.

Frequency of successful cultivation of human breast tumors. Procedures similar to those employed in the isolation and establishment in culture of cell line HBT-3 have been used in attempts to establish continuous cell lines from a series of approximately 100 human breast tumors of varying histological types. Some evidence of cell multiplication was observed in 26 of 32 benign and 59 of 68 malignant tumors. Primary cultures and as many as 5 passages were screened specifically for colonies of refractile epithelioid cells resembling those seen in the HBT-3 line. Colonies meeting these criteria were rare, and when observed they were isolated as de-

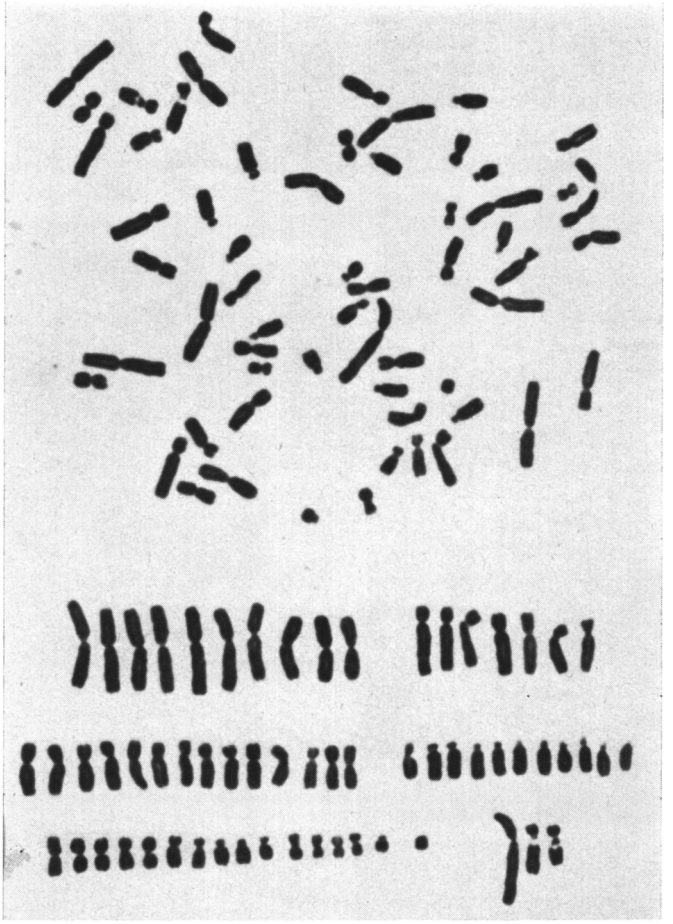

FIG. 6. Karyotype of HBT-3 cell with 61 chromosomes showing 3 markers.

scribed previously. In addition to the HBT-3 cell line, 3 epithelioid cell lines currently at passage levels ranging from 5 to 40 have been isolated and are under investigation.

Cells not meeting the morphological criteria described above were frequently observed. These cells, which almost always resembled the fibroblastic cells seen in Fig. 5, could be subcultured for up to 20 passages, but never gave rise to continuous cell lines.

Discussion. Several lines of evidence indicate that HBT-3 cells are derived from tumor cells present in the original breast carcinoma from which the culture was established. Their epithelioid appearance, their rapid rate of growth continuing over an apparently indefinite number of cell passages, their high cloning efficiency, their karyotype, and their loss of contact inhibition all serve to distinguish HBT-3 cells from other normal cellular elements present in breast tissue and in particular from normal fibroblasts. In addition, it is not likely that HBT-3 cells arose as a "spontaneous" transformant of a normal cell present in the original breast tumor culture, since the original epithelioid colony which gave rise to the HBT-3 cell line was first seen within 7 days after plating of the original tumor tissue. Moreover, this colony was immediately able to give rise to secondary colonies of noncontact-inhibited epithelioid cells when plated under cloning conditions in small chambers.

It seems unlikely that HBT-3 cells arose as a laboratory "contaminant" of the original breast tumor culture with a previously established human tumor cell line such as HeLa. In addition to the rigorous procedures

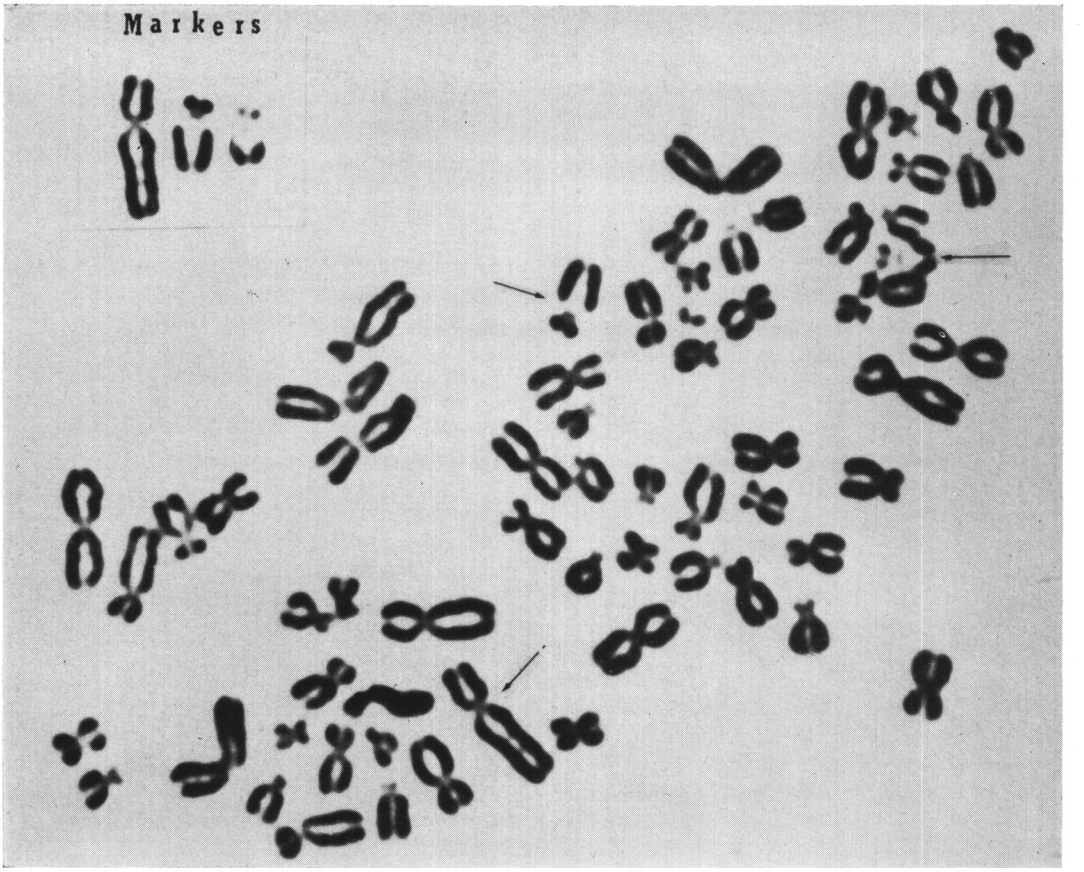

FIG. 7. Metaphase plate from HBT-3 cell with 67 chromosomes. Structural details of 3 marker chromosomes are evident.

routinely employed to eliminate cross contamination of any of our cell lines, HBT-3 cells were isolated and established in a tissue culture laboratory where no human tumor cells were grown except primary breast tumor tissue isolates. Furthermore, this laboratory is part of the Emergency Virus Isolation Facility of the National Cancer Institute, and the probability of contamination from outside sources would be minimized by the carefully controlled air flow patterns and other isolation procedures employed in this building. While it cannot be excluded that the breast adenocarcinoma which gave rise to cell line HBT-3 was not itself a primary tumor, the histological appearance of the tumor cells (Fig. 2) and the fact that the patient has remained free from any other detectable malignancy for a period of over 14 mo since the original tumor was removed both tend to rule out this possibility.

Although the HBT-3 line was established in culture with comparative ease, repeated attempts to culture other breast tumors in this laboratory using either the same or different techniques have not been very successful. The growth of continuous lines such as HBT-3 may be fortuitous, but two of the techniques employed might have been instrumental in developing this cell line when so many other attempts failed. First, the usefulness of collagenase has been reported by Lasfargues and Moore (11). Secondly, the early cloning of suspected tumor cells to separate them from normal tissue cells, which may be present in great excess in most tumors, may have prevented the eventual overgrowth with normal fibroblasts so often seen in other breast tumor cultures. If HBT-3 cells do represent human breast tumor cells in

culture, they might be expected to exhibit many of the virus-associated properties recently demonstrated using *in vivo* derived breast tumor material (3, 6–8). Experiments along these lines are in progress.

Summary. A continuous line of human breast carcinoma cells, HBT-3, was established in culture following collagenase treatment of a mucus producing adenocarcinoma. The cells are epithelioid in appearance, multiply rapidly, have a cloning efficiency of approximately 70%, and exhibit an abnormal karyotype with a mode of 66–69 chromosomes/cell and 3 markers.

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