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Establishment and characterization of a new cell line (VHB-1) derived from a primary breast carcinoma

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Summary. A continuous line of human breast carcinoma cells, VHB-1, was established in culture following collagenase treatment of an infiltrating duct cell carcinoma. The cells displayed an epithelial pattern and multiplied rapidly. Maintained in monolayer culture, the VHB-1 cells exhibited a 30-h doubling time and a plating efficiency of 20%. The cells possessed an abnormal karyotype with a mode of 70–74 chromosomes per cell. The karyotype was heavily rearranged and numerous marker chromosomes were found. Transplantation of the cells into nude mice produced tumors bearing histological resemblance to the original material. The VHB-1 cells contained significant levels of prolactin receptors, were steroid hormone (estrogen, progesterone, androgen, glucocorticoid) receptor positive, and were capable of functional differentiation in vitro. These characteristics make the VHB-1 cell line a suitable model for studying the biological properties of human breast tumors.

Key words: Breast carcinoma cell line – In vitro model

Introduction

Attempts to culture breast tumor cells have so far apparently been less successful with solid tumors than with malignant effusions (Cailleau et al. 1974) and since the first successful long-term culture of a breast tumor (BT 20 cell line) reported by Lasfargues and Ozzelo in 1958, there are still few well-characterized cell lines derived from primary breast carcinoma (Engel and Young 1978). To our knowledge, only one steroid receptor positive cell line derived from a primary lesion has been established (Yamame et al. 1984). The general lack of success that has attended attempts to establish such cell lines lies in the fact that solid tumors are often necrotic and the viability of the cells obtained is usually low, and also contamination by fibroblasts is frequent. Well-characterized cell lines available for study of the pathogenesis of breast cancer are needed for in vitro model systems in multidisciplinary research; in this report we describe the establishment and characterization of an additional positive receptor cell line designated VHB-1 derived from a ductal mammary carcinoma.

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Materials and Methods

Origin and establishment of the cell line

The tumor was removed surgically from a 74-year-old woman, previously treated in January and February 1982 by radiotherapy for breast cancer (T2c N1b M0). Because of local relapse associated with pulmonary metastasis, 18 months later she received tamoxifen plus medroxyprogesterone acetate. As the hormone therapy was no longer efficient she finally underwent a mastectomy in March 1984. She was still receiving hormone therapy at the time of surgery. No lymph node involvement was detected. The tumor was pathologically classified as an infiltrating, moderately differentiated, ductal epithelioma. The patient died from progressive disease in June 1985.

The tumor tissue was received in the laboratory 15 min after surgery, and fatty tissue was separated from the firmer part of the tumor. The tumorous material was then rinsed with Earle's balanced salt solution, minced using 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 cleared of most of the fibroblasts by a sedimentation fractionation procedure according to Hiratzuko et al. (1982); the culture was then substained in 50-ml polystyrene tissue culture flasks (Primaria, Falcon Becton Dickinson Oxnard Calif., USA). The medium used was Earle's minimal essential medium (MEM, Seromed Biochrom KG, Berlin, Germany) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 250 µg/ml amphotericin B, 10% fetal calf serum, and 5 µg/ml insulin.

Growth rate determination

To study the growth rate of VHB-1 cells 1.5×10^5 cells/2 ml liquid medium were seeded in Leighton tubes (3.52 cm^2). After detachment

by trypsinization, Coulter counter cell counts were determined daily without refeeding. Doubling times were determined during the log phase of the growth.

The cell plating efficiency on plastic was studied by seeding 1×10^3 to 5×10^3 cells in 6-cm Petri dishes. Then 15 days after plating, plastic adherent colonies were washed gently with phosphate-buffered saline (PBS, 0.15 *M* NaCl, 0.1 *M* phosphate, pH 7,2) then stained and fixed simultaneously in methanol formalin crystal violet solution.

Growth in semisolid medium

For anchorage independent cloning assays, the medium used for cell cultivation was gelled with agarose to a final concentration of 0.56% for the bottom layer and 0.36% for the plating layer. Petri dishes (6 cm) containing 2.5×10^4 VHB-1 cells in agar medium were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Colonies were counted at 15–20 days after plating using an inverted microscope.

Tumorigenicity

Athymic nude mice were used to estimate malignancy of the VHB-1 cells. The animals received s. c. injections of 3×10^6 cells in PBS. The animals were also stimulated by one injection of 1 mg estradiol (oestradiol-retard, Theramex, Monaco) per month.

Study of epithelial nature of the cells

Tumors for light microscopy were fixed in 10% buffered formalin. Sections from paraffin-embedded tissue were cut at 5 µm and stained with H & E or Alcian blue (pH 2.5) for mucin detection.

Glycoproteins isolated from human milk fat globule membrane, designated epithelial membrane antigen (EMA) were detected immunohistochemically with a commercially available monoclonal antibody (anti-EMA antibodies, clone E 29, Dako Corporation, Santa Barbara, Calif., USA) using a peroxidase-antiperoxidase (PAP) technique on paraffin-embedded induced tumors.

The intermediate filaments of cytokeratin were stained on formalin-fixed, paraffin-embedded tissue sections by a PAP technique using a monoclonal antibody which reacts with human keratin 55–57 kd (clone KL_1, Jackson Immunoresearch Laboratories Inc, Avondale, Pa., USA).

The intermediate filaments of vimentin were visualized using a monoclonal antibody (clone V9, Amersham, Buckinghamshire, UK).

Karyotype analysis

The cells were treated with 0.04% colcemid for 2 h at 37 °C, placed in hypotonic 0.3% KCl sodiumcitrate solution for 10 min, then fixed with methanol acetic acid solution (3:1). R banding was performed according to Dutrillaux and Viregas-Pequignot (1981).

Mycoplasm and enzyme determinations

Mycoplasm contamination was detected by DNA staining using the mykoplasmentest kit from Seromed (Biochrom KG, Berlin, Germany). Phenotypes of lactate dehydrogenase (LDH), and glucose-6-phosphate dehydrogenase (G6PD) isoenzymes were determined electrophoretically using kits from Helena Laboratories (Beaumont, Tex., USA). Catechol-*o*-methyltransferase (COMT) and estrogen-2-hydroxylase were determined according to Hoffman et al. (1979).

Hormone receptors

Estrogen and progesterone receptors analyses were performed on the patient's original tumor in 1984 using classical DCC methods (EORTC Breast Cooperative Group 1979; Duffy and Duffy 1979) and prolactin receptors were assayed on microsomal fractions (Peyrat et al. 1984).

In the cell line, steroid hormone receptors were measured by whole cell assays in monolayer cultures (Olea-Serrano et al. 1985; Steiner and Wittliff 1985). The steroids used were ^{3}H-estradiol 90 Ci/mmol and ^{3}H-dihydrotestosterone 128 Ci/mmol from New England Nuclear (Boston, Mass., USA) and ^{3}H-org-2058 47 Ci/mmol and ^{3}H-triamcinolone acetonide 30 Ci/mmol from Amersham (Buckinghamshire, UK).

All the unlabeled steroids were from Steraloids Inc (Pawling, NJ, USA). Cultures were performed at 37 °C in T-75 flasks. The medium used was Earle's MEM supplemented with L glutamine (0.6 mg/ml) penicillin (100 IU/ml) and 10% inactivated fetal calf serum. After 4 days of culture, a fixed number of cells grown in multiwell dishes were pulsed with ^{3}H-steroids for 30 min in serum-free medium at concentrations ranging from 0.25 to 20 n*M*. Additional dishes were filled with the same concentrations of labeled steroids plus a 200-fold excess of the corresponding unlabeled steroid. After incubation the medium was removed, the cells washed with ice-cold 0.9% saline, and bound steroids were extracted from the monolayer by incubation in ethanol at room temperature. After counting aliquots of ethanol, specific ^{3}H-steroid uptake was calculated from the difference in incorporated radioactivity after incubation in the absence or presence of excess of unlabeled steroid. Binding parameters were analyzed by the method of Scatchard (1949); maximal binding capacities were expressed in Femtomoles per 10^6 cells and the dissociation constants in nanomoles per liter. Prolactin receptors were measured on cell membranes using 200000 cpm ^{125}I human growth hormone (80 µCi/µg) as tracer (Peyrat et al. 1984). Specific binding capacities were expressed in femtomoles per miligram of protein.

Induced proteins

Two milk proteins were studied in the culture media; firstly α-lactalbumin assayed using antisera raised against pure α-lactalbumin (Peyrat et al. 1986), the sensitivity of the assay was 0.2 ng ml of medium; secondly the sweat α-2globulin described by Jirka (1968) which is present in a number of external secretions. This protein has been extensively studied by Haagensen (1981) and termed GCDFP15 (Gross Cystic Disease Fluid Protein). We have purified this product from breast cystic fluid of patients with breast cyst disease, and raised antisera against the protein (Vandewalle et al. 1986); the sensitivity of the radioimmunoassay was 5 ng ml of medium.

Results

Establishment of culture and morphological features

Explantation of material from patients with mammary cancer resulted in yield of only 25 successful subcultures from 47 experiments; 24 of these died after the first transplantation and only 1 gave a stable line of cells. The day after seeding a few elongated branching fibroblasts formed network-like complexes but in the culture there were also rather large, scattered, epithelium-like cells which differed sharply from the surrounding fibroblast-like cells. These cells had very large nuclei and a relatively small mass of cytoplasm. As cultivation proceeded the fibroblast-like cells were gradually displaced by vigorously proliferating islands of epithelium-like cells, and in subsequent passages

the fibroblasts disappeared. The cells were irregular in shape and demonstrated intracytoplasmic vacuoles and aggregation of chromatin granules. They revealed neoplastic and pleomorphic features such as irregularities in size and shape of nuclei and nucleoli (Fig. 1). By electron microscopy, cells showed ultrastructural features of epithelium (Fig. 2). Culture tests for mycoplasm at the 20th and the 89th passages were repeatedly negative.

Growth characteristics

Growth curves of VHB-1 cells in liquid medium are shown in Fig. 3. In the exponential growth phase the average doubling times (DT) at the 25th and the 87th passages were estimated to be 37 and 31 h, respectively, The beginning of the plateau (day 4) corresponded to a cell density of 4.2×10^5 cells/cm^2. Depletion of exogenous insulin did not significantly modify the growth of VHB-1 cells (DT = 30 h). VHB-1 cells grew uninterruptedly for over 30 months.

Clonal studies

In order to check the tumoral nature of the VHB-1 cells, we tested their ability to grow in agar. After 15 days of culture in semisolid agar, cells presented a 4% cloning efficiency. In a liquid medium, the plating efficiency as defined by Puck and Marcus (1955) reached 20%.

Heterotransplantation

The s.c. heterotransplantation of the VHB-1 cells into 6 nude mice continuously stimulated with estradiol led to the formation of 6–10 mm palpable tumors in 4 animals within 8 weeks. We always failed to induce tumors in nonstimulated animals.

The tumors obtained were soft, grayish white. The microscopic appearance mimicked the poorly differentiated solid masses of the original primary tumor (Figs. 4 and 5a). There were some lacunae throughout the cell masses and the cells sometimes showed mucus-secreting vacuoles which were visualized using Alcian blue stain in about 5%–10% of the cell population (Fig. 5b).

Nearly 20% of the cells were immunoreactive for EMA with granular cytoplasmic staining of variable intensity (Fig. 5c). Furthermore, nearly all the tumoral cells exhibited diffuse staining with cytokeratin monoclonal antibodies (Fig. 5d). Using anti-vimentin antibodies no staining was observed in any epithelial cells.

Chromosome analysis

A total of 65 metaphase plates corresponding to cultures from the 20th and the 87th passages were selected for chromosomal study. No shift in modal chromosome number was observed in these two populations, which showed a predominance of cells with a chromosome count of 72; 91% of those cells demonstrated chromosome numbers of 70–74 (Fig. 6).

R banding chromosome analysis revealed a heavily rearranged human karyotype. Using this staining, which highlights telomeric parts of the chromosomes, only 39 normal chromosomes could be identified (Fig. 7). The other chromosomes termed as markers might had resulted either from intrachromo-

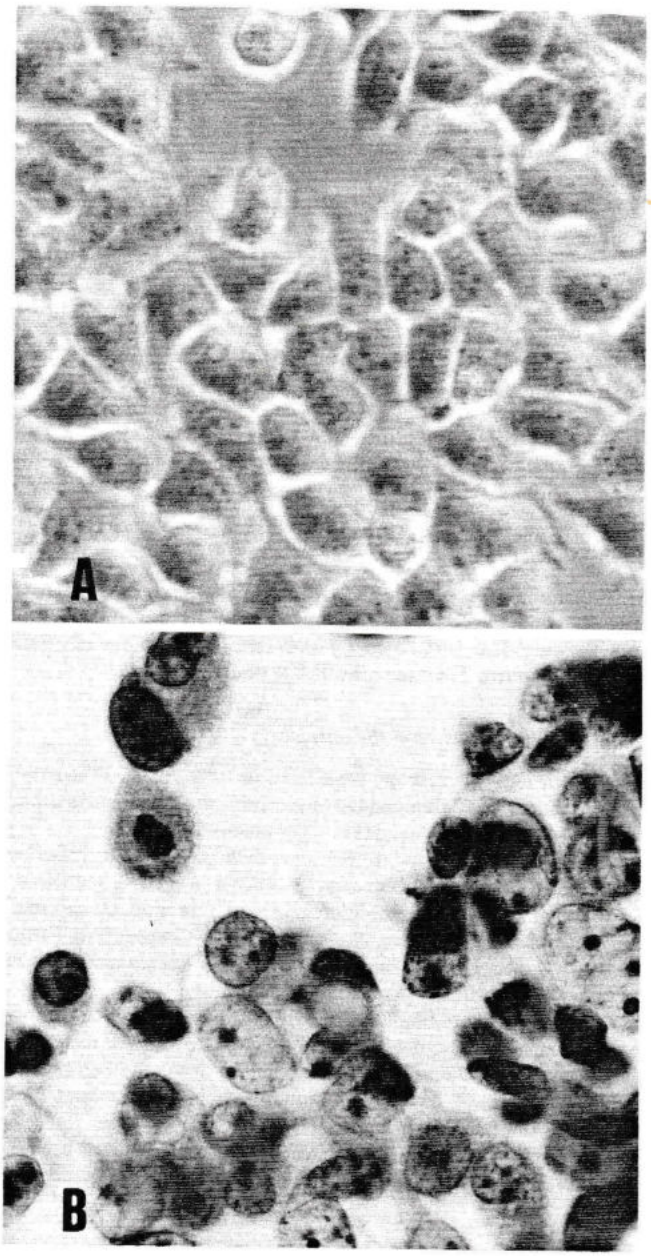

Fig. 1. Light micrograph of VHB-1 cells on the 3rd day of culture (passage 87). A: monolayer cultures, phase contrast (× 320); B: Papanicolaou staining (× 1 000) showing anisonucleosis, nucleoli, mitotic figures, and intracytoplasmic vacuoles

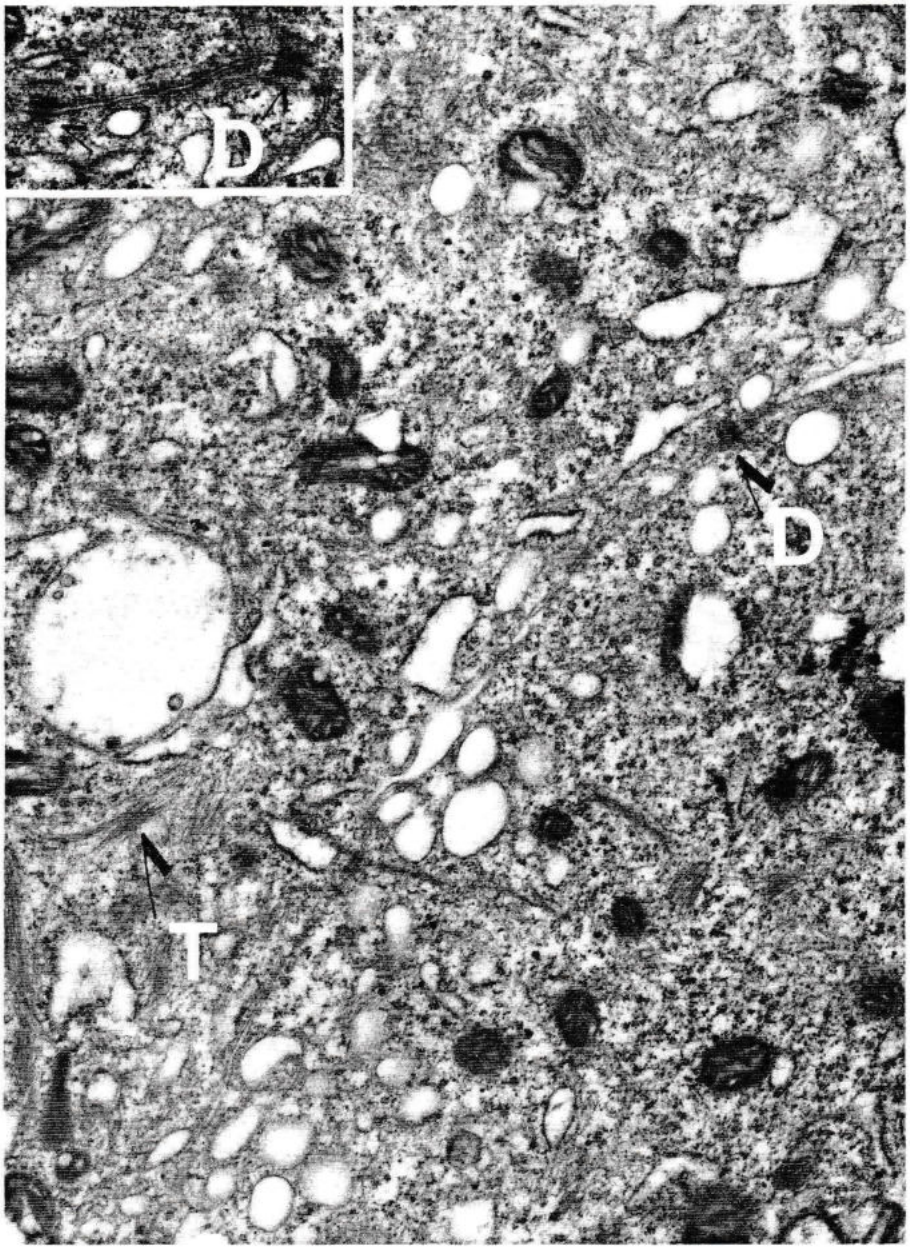

Fig. 2. Electron micrograph of VHB-1 cells (passage 87) showing characteristic bundles of tonofilaments *(T)* and desmosomes *(D)* (× 8000). Two other typical desmosomes are represented in the insert (× 10000)

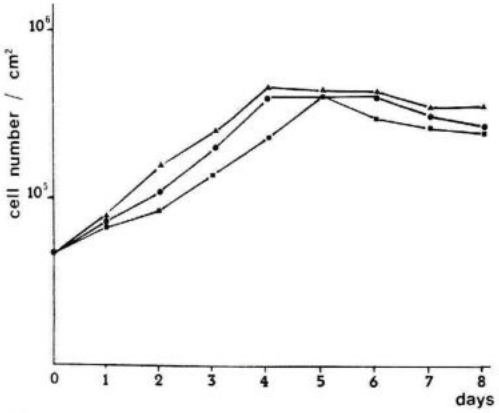

Fig. 3. Growth curves of VHB-1 cells. 25th passage: ■; 87th passage: ▲ with or ● without insulin in the culture medium. Each point represents the mean of 12 tubes

somic rearrangements (i.e. , isochromosomes, ring chromosomes) or interchromosomic rearrangements. Large size chromosomes seemed more frequently rearranged than the others, but due to the great number of abnormal chromosomes, it was impossible to ascertain if the modifications resulted from two rearrangements or more. Furthermore, besides structural rearrangements leading to aberrant chromosomes, numerical abnormalities were observed in the following groups of chromosomes: group A: nullosomy 1 and 2, monosomy 3; group B: trisomy 4; group C: nullosomy 10, tetrasomy 11, monosomy X; group E; monosomy 17; group F: monosomy 20, and group G: trisomy 21, monosomy 22.

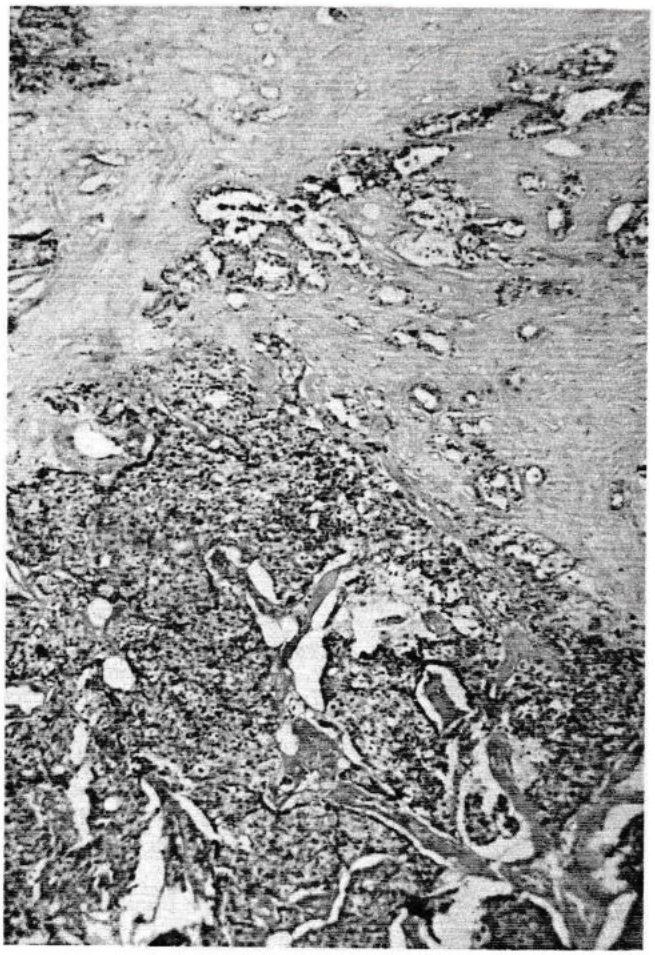

Fig. 4. Low power view of the original tumor from which cell line VHB-1 was derived showing irregular acinar structures in a fibrous stroma and solid masses. The tumor was a moderately differentiated ductal carcinoma (H&E ×63)

Enzyme determination

Isoenzyme determination revealed LDH of human phenotype. All LDH in the VHB-1 cell line was LDH 5. Type B isoenzyme mobility pattern for G6PD or slow migrating band was constantly found in VHB-1 cells. Enzymatic activities of COMT and estrogen-2-hydroxylase were respectively 1 350 and 30 pmol of 2-methoxyestradiol formed per 20 min/mg protein.

Differentiation features

Hormone receptor. The patient's original tumor was weakly estrogenreceptor positive (28 fmol/mg protein) and progesterone receptor negative, furthermore prolactin receptor levels were at the threshold of detection i.e., 1.5 fmol/mg protein.

VHB-1 cells were incubated with increasing concentrations of ^{3}H-steroids to measure the binding reaction parameters. As found by Olea-Serrano et al. (1985), analysis of the data according to Scatchard gave patterns indicative of two classes of binding sites, a high-affinity class of limited capacity and a low-affinity class of high capacity; the high-affinity class of binding sites corresponds to type I binding sites defined as classical receptors. The dissociation constant (Kd) of the binding reactions of ^{3}H-steroids to the high-affinity sites and the binding capacities of these sites are shown in Table 1. They are in good agreement with values currently ascribed to receptors (Olea-Serrano et al. 1985; Steiner and Wittliff 1985). Prolactin receptors determined by conventional biochemical methods were weakly positive in cell membranes.

Table 1. Hormone binding characteristics of VHB-1 cells at the 95th passage

Type of receptor	Binding capacity[c]	Dissociation constant (Kd)
Estrogen	72± 31[a]	0.5±0.3
Progesterone	220±100	0.6±0.4
Glucocorticoid	25± 14	2.2±1.3
Androgen	123± 35	1.4±0.4
Prolactin	12± 4[b]	

[a] The binding capacities of steroid receptors are expressed in fmol/10^6 cells, the dissociation constants are expressed in 10^{-9} *M*
[b] The binding capacity is expressed in fmol/mg protein
[c] Mean values ±SD of 3 different experiments

Table 2. Progesterone binding of VHB-1 cells following estradiol stimulation for 72 h

	Cell number per flask	specifically bound 3H-org-2058 (fmol per 10^6 cells)
Control cells	977800±38400[a]	185±6.4
Estradiol-treated cells (10^{-8} *M*)	1219340± 7930	238±7.1

[a] Mean ± SD of 3 different experiments (passage 87)

Hormone responsiveness. Following estradiol stimulation of VHB-1 cells by 10^{-8} *M* estradiol over 3 days, synthesis of progesterone receptors was noted and the ^{3}H-org-2058 incorporated per 10^6 cells was about 30% more than the control values. At the time there was an approximate 20% increase in total cell number in estradiol-treated cells over controls (Table 2).

Induced proteins. When the cells were cultivated in MEM without fetal calf serum but containing 5 µg/ml insulin, 30 µg/ml transferrin, and 7.5 ng/ml fibronectin we were able to detect alpha-lactalbumin and GCDFP-15 in the 72-h-culture media. After estradiol stimulation (10^{-8} *M*) both levels dropped compared to base levels (Table 3).

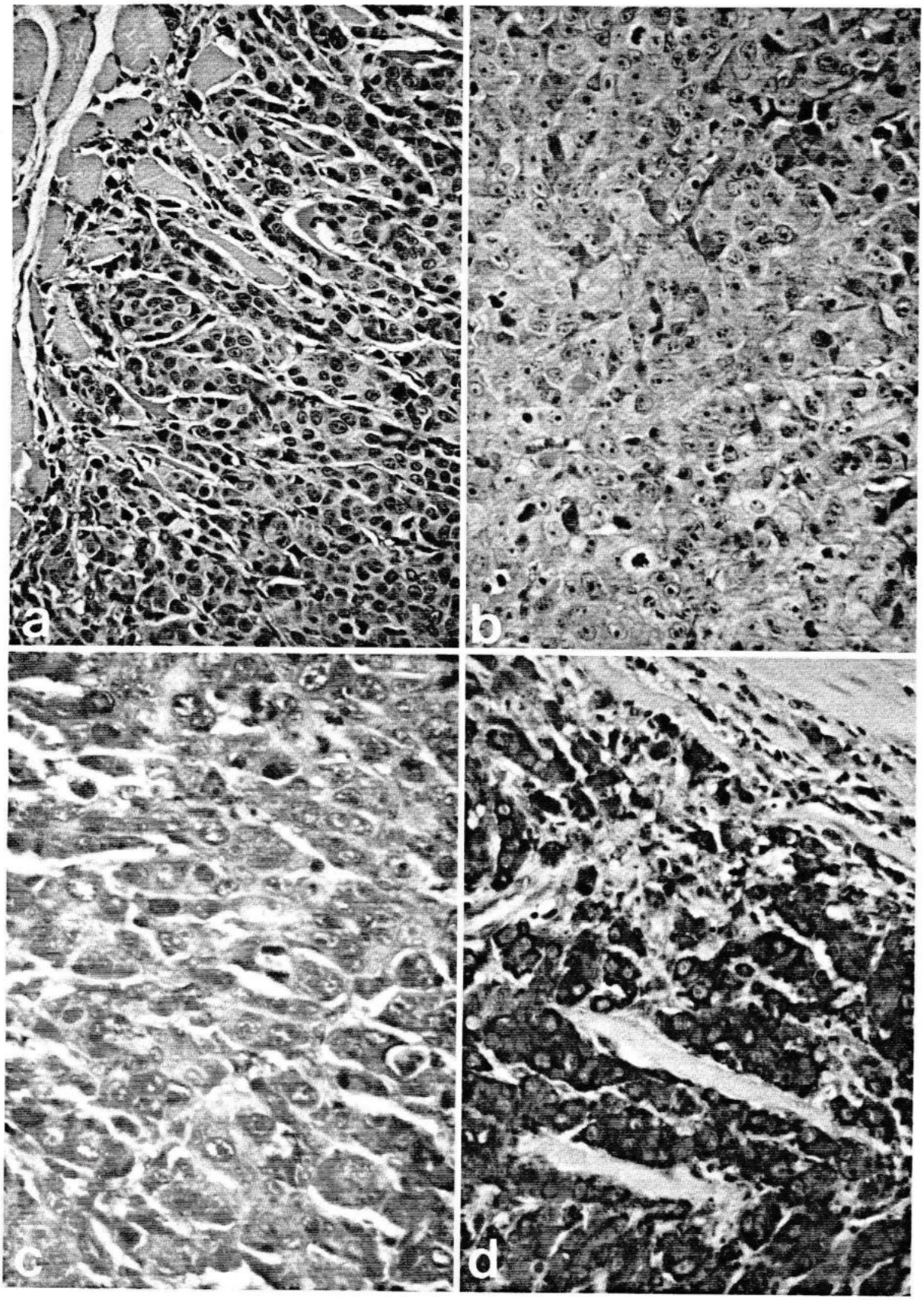

Fig. 5 a–d. Tumor induced by VHB-1 cells. **a** Tumor shows a poor differentiation and infiltration of muscle by carcinomatous cells (H&E $\times 250$). *b* Alcian blue stain shows some positive mucin secreting cells ($\times 400$). *c* Formalin-fixed, paraffin-embedded tissues sections stained with monoclonal anti-epithelial membrane antigen. Cytoplasmic staining is shown in nearly 20% of the cells ($\times 400$). **d** Formalin-fixed, paraffin-embedded tissue section stained with monoclonal anti-keratin. Intense cytoplasmic staining is present in nearly all carcinoma cells in contrast to unstained striated muscle cells (hematoxylin counterstain $\times 400$)

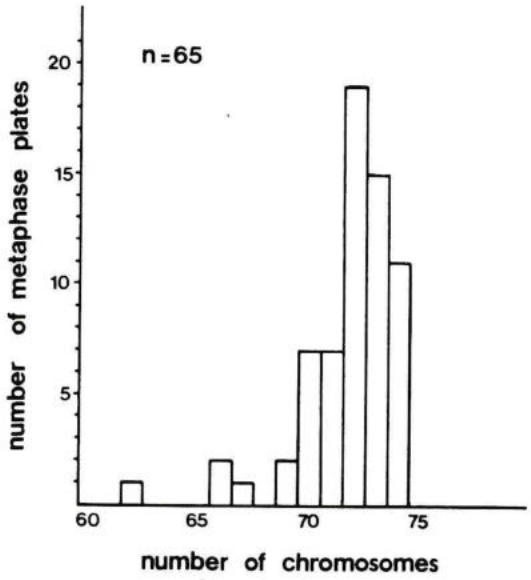

Fig. 6. Chromosomal number distribution in a random count of 65 VHB-1 cell metaphases. The modal number was 72

Table 3. Proteins secreted in the 72-h culture media by VHB-1 cells (passage 87)

	α-Lactalbumin	Gross Cystic Disease Fluid Protein
Controls cells	0.52 ± 0.04	9.8 ± 0.5
Estradiol-treated cells (10^{-8} *M*)	0.27 ± 0.02	5.5 ± 0.3

Values (mean ± SD, $n = 6$) are expressed in ng per 10^6 cells

Discussion

The aim of development of tumor cell lines is the creation of an in vitro representation of malignant cells in the primary tumor, it seems thus more logical to begin with tumor cells from the original tumor site. To avoid contamination with fibroblasts, which is one of the major difficulties in establishing cell lines from solid mammary tumors, we used the simple method of Hiratsuka et al. (1982) which yields enriched epithelial cell cultures as a result of the differential rate of settling between fibroblasts and epithelial cells, and we used primaria culture flasks known to inhibit fibroblast proliferation. As cultivation proceeded the fibroblasts were gradually displaced by vigorously proliferating epithelial cells. In this way, among the 47 breast tumors processed, we succeeded in developing an epithelial cell line termed VHB-1. A long-term and stable proliferation then took place over more than 130 successful passages during 30 months. The VHB-1 cell population DT was 31–37 h, saturation density was 4.2×10^5 cells/cm^2, and their cloning efficiency was very high. These values are in agreement with those found in other cell lines established from primary tumors (Yamame et al. 1984; Plata et al. 1973). The chromosome distribution showed a single population of hyperploid cells with a stable modal chromosomal number of 72 throughout successive cultures, the apparent stability noted both in chromosomal number and in number and morphological features of rearranged chromosomes suggested that the malignant feature did not come from long-term in vitro cultivation.

Specific cell organelle characteristics of in vitro tissue may be retained after long-term growth in vivo (Buchring and Hackett 1974), thus the ultrastructure of cells in culture may reflect the tissue of origin. Electron microscopic examination of VHB-1 cells showed the presence of desmosomes and bundles of tonofibrils that are characteristic structure markers identifying epithelial cells.

The microscopic features of the induced tumors were histologically similar to a poorly differentiated adenocarcinoma with moderate mucin secretion. Epithelial membrane antigen, which is a highly effective marker for establishing the epithelial nature of neoplastic cells (Pinkus and Kurtin 1985) was detected in the tumor cells, conversely, they reacted positively with cytokeratin monoclonal antibodies and it is known that keratin filaments are present in almost all epithelial cells (Franke et al. 1979). Moreover negative staining with vimentin monoclonal antibodies supplied evidence that the VHB-1 cells were not cells of mesenchymal derivation. The question as to whether these epithelial tumor cells have retained some of the functional activities that would characterize their mammary origin was investigated by determination of the secreted proteins alpha-lactalbumin and GCDFP-15. The presence of detectable levels of these proteins indicated that the cells had not lost their differentiation characteristics.

Using whole cell assays, which give a valuable estimation of the total unsaturated receptor contents, VHB-1 cells were shown to contain estrogen, progesterone glucocorticoid, androgen Steroid receptor capacities were stable throughout cell cultures. Prolactin receptor capacities were at the threshold of detection at early cultivation and grew till the 50th passage. They then stabilized and were weakly positive. Estrogen receptors were functional and responsive to estradiol stimulation since progesterone receptor synthesis was induced. Most of the cells containing estrogen and progesterone receptors are derived from pleural effusions or ascites (Engel and Young 1978). There is, to our knowledge, only one recent report of the presence of estrogen and androgen receptors in a cell line derived from a solid breast tumor (Yamame et al. 1984). The VHB-1 cells

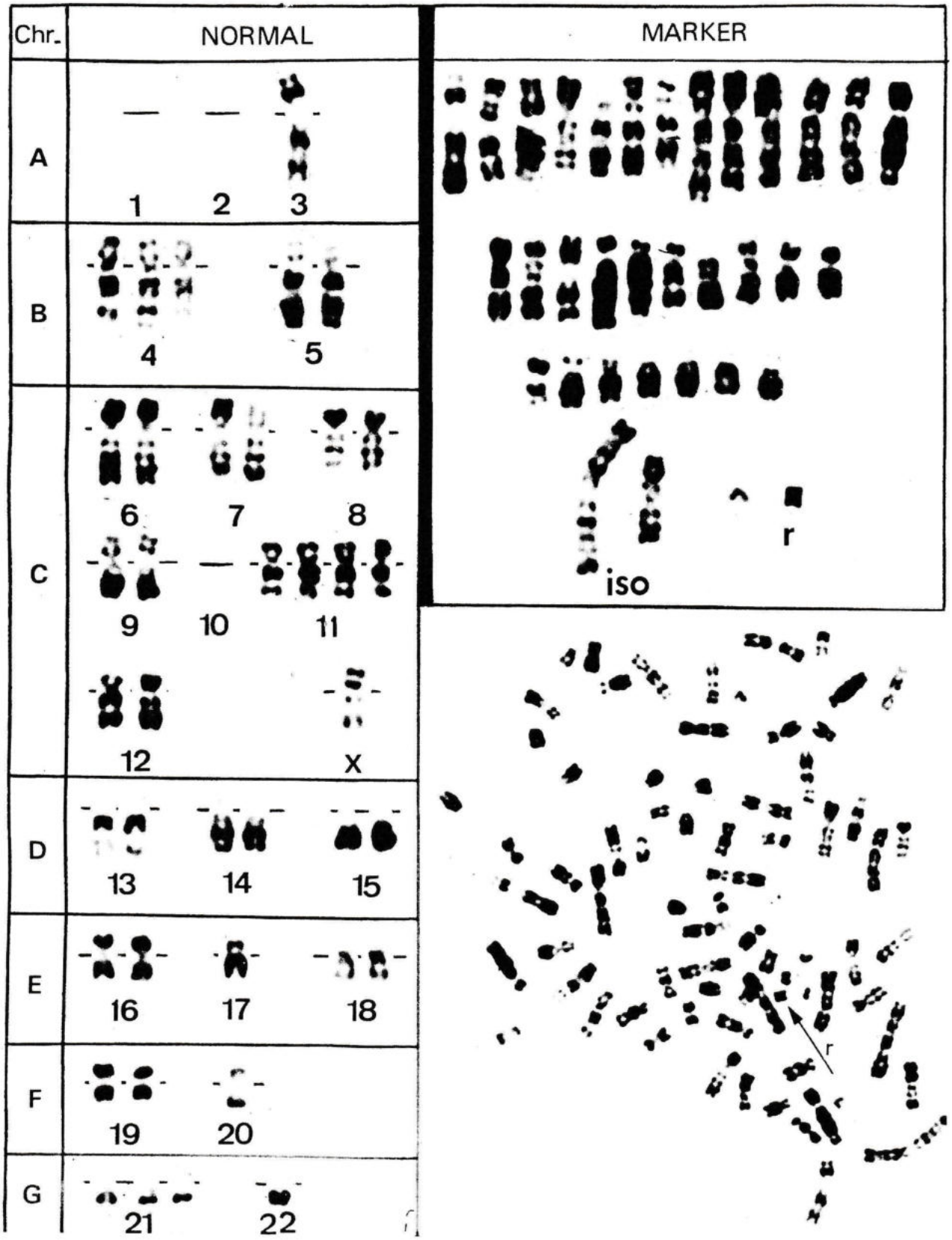

Fig. 7. R-banded karyotype of VHB-1 cells. Using this staining only 39 normal chromosomes out of the 73 could be identified. The other rearranged marker chromosomes were numerous. *Iso*: Isochromosome, *r*: ring chromosome

are able to produce differentiation proteins, which supplies additional evidence that they have retained functional activities. These results indicate that VHB-1 cells could be used for hormone stimulation studies and for establishing structure-activity relationships. The need for additional cell culture material is obvious in oncogene studies. As several reports have described the expression of oncogenes analogous to retroviral oncogenes in established cell lines from a variety of tumors, experiments are in progress to analyze possible genomic rearrangements and variations in the expression of several oncogenes. Furthermore, experiments are currently being carried out to evaluate the transforming capability of VHB-1 DNA on NIH 3T3 cells.

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