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Two New Human Tumor Cell Lines Derived from Squamous Cell Carcinomas of the Tongue: Establishment, Characterization and Response to Cytotoxic Treatment

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Abstract—*Two new permanent cell lines derived from squamous cell carcinomas of the tongue, CAL 27 and CAL 33, have been established in culture. Both cell lines were isolated in standard culture media without epidermal growth factor or fibroblast feeder layer to avoid obtaining clones of more differentiated cells. Analysis of the morphology, ultrastructure, karyotype and immunohistochemical properties of these two cell lines demonstrated that they are both well characterized, uncontaminated by HeLa cells, and do in fact correspond to transformed epithelial cells that have conserved certain characteristics of the original Malpighian epithelium. CAL 27 and CAL 33 have relatively long doubling times (35 and 43 h respectively). Their response to 14 drugs used for cancer chemotherapy was evaluated by a short term assay based on tritiated thymidine incorporation after exposure to the drugs. CAL 27 was more resistant than CAL 33 in all cases but one. Although cytogenetic examination revealed both lines to be malignant, neither CAL 27 nor CAL 33 produced colonies in soft agar; both lines were tumorigenic after inoculation into nude mice. This study clearly demonstrates the diversity of cancers of a given histologic form, in agreement with the diversity noted previously* in vivo. *Isolated without the use of any selection criteria, these cell lines constitute appropriate models for the study of human tumors.*

INTRODUCTION

THE ESTABLISHMENT of permanent cell lines involves no problems for certain human neoplasms such as malignant melanoma and adenocarcinoma of the colon or kidney. A wide variety of models required for investigations is available for these histologic forms.

Some 20 or so cell lines of squamous cell carcinomas of the upper aerodigestive tract have already been well characterized [1–5].

The CAL 27 and CAL 33 cell lines were established in 1982 and 1983 respectively in our laboratory from two squamous cell carcinomas of the tongue after 100 attempts to culture biopsies of this type of cancer. In addition to study of their morphology, ultrastructure, karyotype and immunological properties, their response to various cytotoxic drugs used for cancer treatment was evaluated.

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MATERIALS AND METHODS

Clinical history and tissue culture

Both cell lines were established in culture and maintained in Dulbecco's modified minimum essential medium with Earle's salts (Boehringer, France SA) supplemented with 2 mmol L-glutamine, 10% fetal calf serum, 400 U/ml penicillin, and 200 μg/ml streptomycin. Cells were stored in an incubator at 37°C in a 5% CO_2 atmosphere.

CAL 27

Patient G presented on 30 March 1982 with a lesion of the middle third of the oral tongue. Before any treatment, a fragment was excised under sterile conditions, placed in sterile culture medium, and brought to the laboratory. The fragment was then cut into 1–2 mm dia. explants which were placed on the bottom of a screw-top culture flask (Falcon, Los Angeles, CA) and covered with a nutritive medium. The flask was then placed in a CO_2 atmosphere incubator at 37°C.

CAL 33

Patient B presented on 19 January 1983 with a lesion of the tongue. Before any therapy, a fragment was removed surgically and cultured in the form of explants in a sterile culture flask, as described for CAL 27.

Table 1 summarizes the clinical data for these two patients as well as the salient points of their disease course.

Measurement of the doubling time

At the 50th passage, a given number of cells were plated in several Falcon plastic flasks containing complete culture medium. Flasks were placed in an incubator at 37°C with 5% CO_2 in air. Cells were harvested with a solution of 0.05% trypsin in phosphate-buffered saline and were counted at regular intervals, starting 24 h after plating, during 11 days.

Electron microscopy

Cells in monolayer culture were washed with a 2.5% glutaraldehyde solution in cacodylate buffer and covered with this same solution for 2–3 min. The cell layer was scraped with a rubber policeman to obtain groups of cells. After 1 h of fixation, the cells were washed with the same buffer and postfixed with 1% osmium tetroxide. The residue was dehydrated, embedded in Epon, and polymerized for 48 h in an oven. Ultrathin sections were prepared using an LKB Nova ultramicrotome, stained with uranyl lead acetate, and observed with a Hitachi HU 12A microscope.

Immunochemical studies

After fixation in Bouin's solution, the biopsy material was embedded in Paraplast (Crilou, France) and cut into 4 μm thick slices. The cells cultured on glass slides were fixed in a solution of 50 ml of 3% hydrogen peroxide and 200 ml of absolute methanol for 20 min. The Dako Pap K518 kit (SEBIA, France) was used as an indirect immunoassay (peroxidase antiperoxidase), revealing the immune complex (antikeratin polyclonal antibody) as a brick brown color (diaminoethylcarbazole).

Chromosome studies

Cytogenetic examination of CAL 27 and CAL 33 cells was performed at around the 10th passage. Exponentially growing cells were treated with colchicine (Seromed, Biopro) for 2 h at a final concentration of 3.3 μg/ml. The cells were then trypsinized, washed, and treated for 45 min with a hypotonic solution (0.038 M KCl in distilled water). Cells were then fixed in a mixture of acetic acid and methanol (1:3), and dropped onto grease-free, cooled slides. The R bands were obtained by careful thermal denaturation of the chromosomes according to the method of Dutrillaux and Lejeune [6]. The slides were then stained with 4% Giemsa solution for chromosome counting and examination.

Chemosensitivity

Logarithmically growing cells were trypsinized and suspended in culture medium supplemented with 10% fetal calf serum. After adjustment to a concentration of $2–4 \times 10^4$ cells/ml, the suspension was distributed among 96 microtiter plate wells ($2–4 \times 10^3$/well) (Greiner).

After 72 h of incubation at 37°C, the medium was discarded. The drugs in solution in 199 medium (Boehringer, Mannheim, F.R.G.), supplemented with fetal calf serum were then added (see Fig. 7 for the nature and concentration of drugs):

ADM: adriamycin (Adriblastin; Roger Bellon, Neuilly sur Seine, France),
ACTD: actinomycin D (Cosmegen; Merck Sharp & Dohme-Chibret, Paris, France),
ARAC: 1-β-D-arabinofuranosylcytosine (Aracytine; Upjohn, Paris, France),
BLM: bleomycin (Bleomycin; Roger Bellon; Neuilly-sur-Seine, France),

Table 1. *Patient characteristics*

Tumor cell line	Patient	Therapy
CAL 27	Male 56 years Oral tongue Poorly differentiated squamous cell carcinoma Died 6 months after diagnosis	Radiotherapy after tumor biopsy Partial glossectomy and neck dissection 21 nodes: 4 N+ squamous cell carcinoma 1 N+ papillary thyroid carcinoma 1 chemotherapy course
CAL 33	Male 69 years Oral tongue Moderately differentiated squamous cell carcinoma Died 13 months after diagnosis	Radiotherapy after tumor biopsy Salvage partial glossectomy Salvage interstitial curietherapy

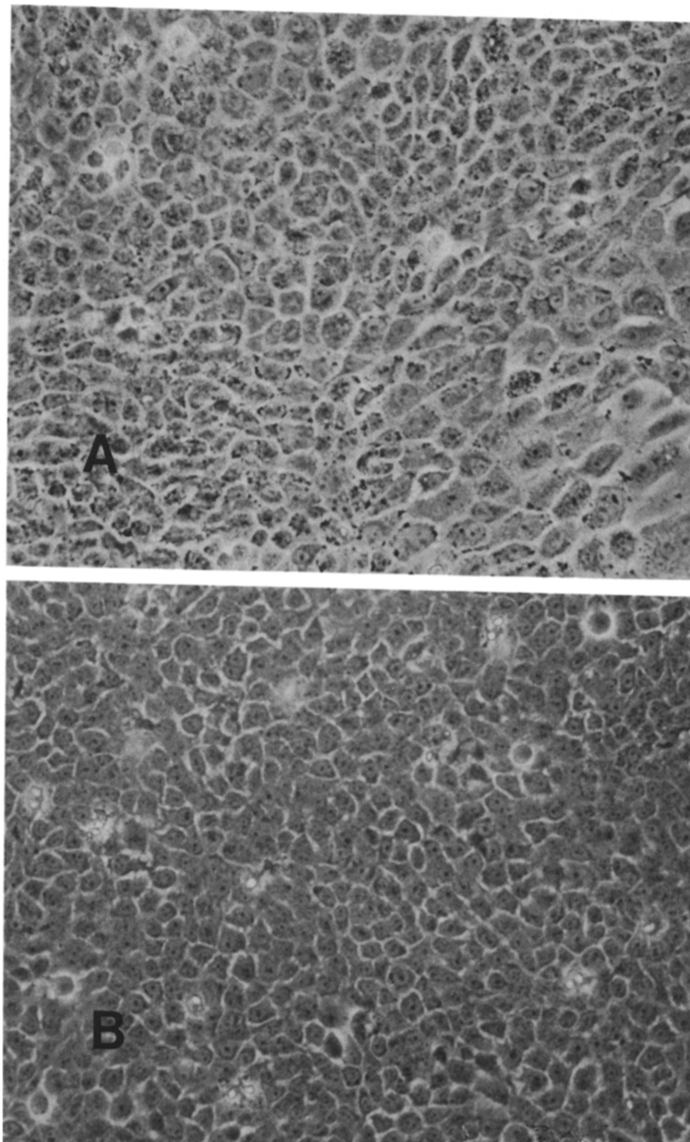

Fig. 1. Photomicrographs of the two squamous cell carcinoma lines (× 100): (*a*) *CAL* 27; (*b*) *CAL* 33.

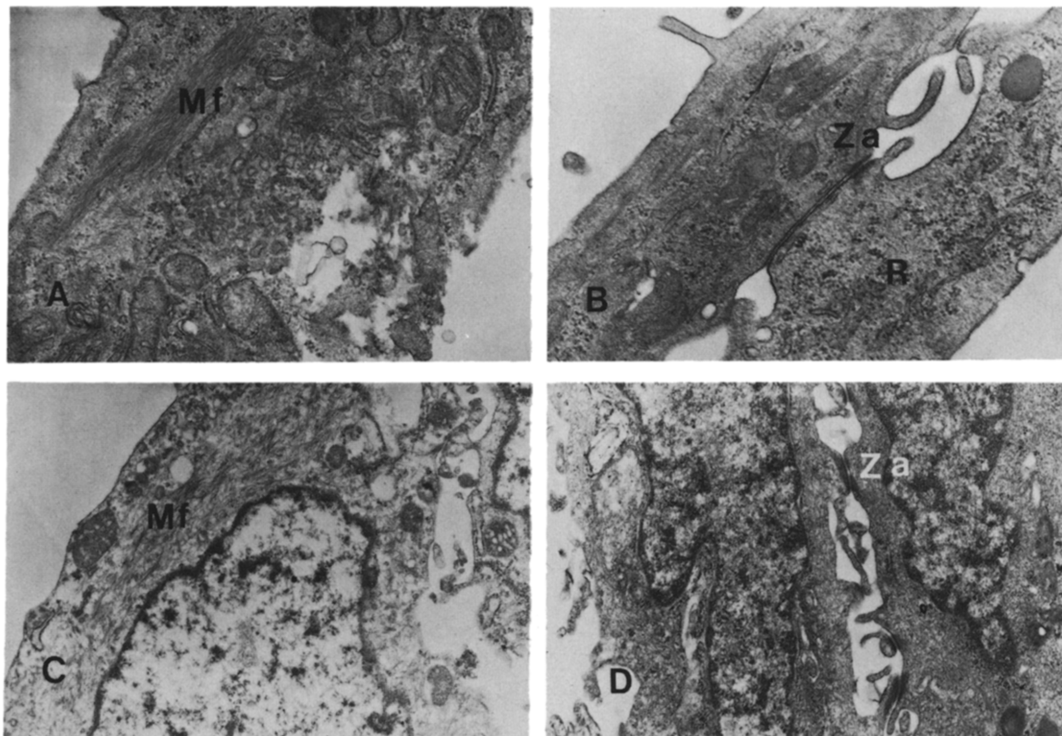

Fig. 2. *Electron micrographs of the two squamous cell carcinoma lines. CAL* 27: (*a*) *microfilaments* (*MF*) (× 17,000); (*b*) *zonula adherens* (*ZA*) *and free ribosomes* (*R*) (× 20,000). *CL* 33: (*c*) *microfilaments* (*MF*) (× 8000); (*d*) *zonula adherens* (*ZA*)(× 12,000).

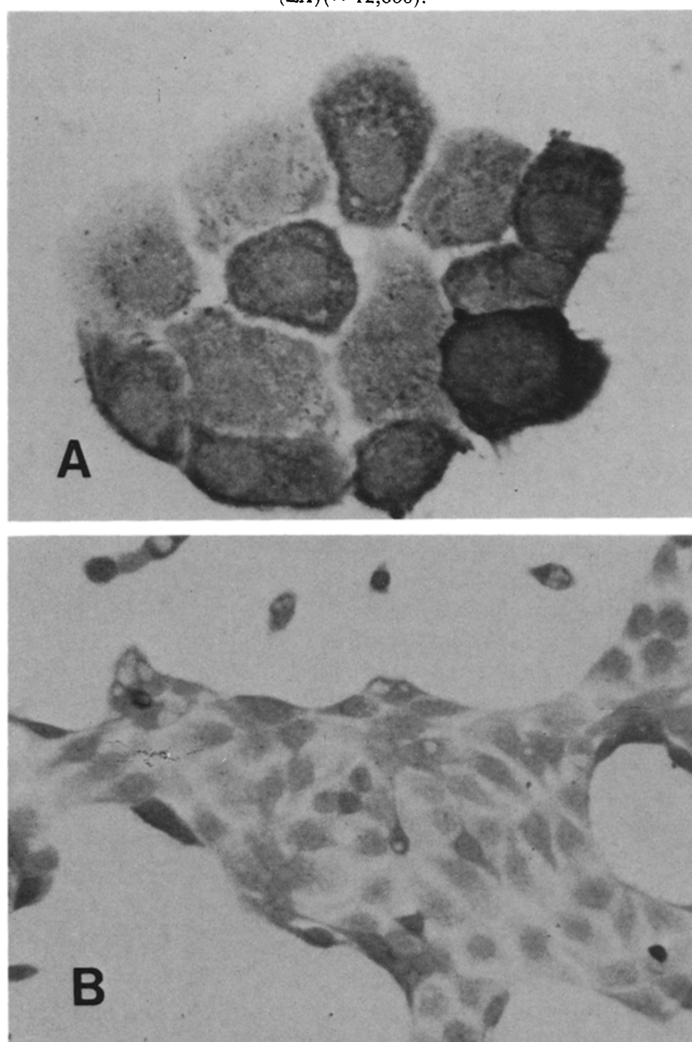

Fig. 3. *Immunopereoxidase staining with antikeratin antibodies*: *CAL* 27 *and CAL* 33 *in culture on glass slides*: (*a*) *CAL* 27 *cells* (× 1000); (*b*) *CAL* 33 *cells* (× 250).

CPA: an active derivative of cyclophosphamide (Asta 7557; kindly supplied by Asta, Hannover, F.R.G.),
CDP: *cis*-platinum (Cisplatyl; Roger Bellon, Neuilly-sur-Seine, France),
CCNU: 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (Bélustine, Roger Bellon, Neuilly-sur-Seine, France),
5-FU: 5-fluorouracil (Fluoro-Uracyle; Roche; Neuilly-sur-Seine, France),
MMC: mitomycin (Ametycine; Choay, Paris, France),
MTX: methotrexate (Ledertrexate; Lederle; Oullins, France),
VDS: vindesine sulfate (Eldesine; Lilly; Saint-Cloud, France),
VLB: vinblastine (Velbe; Lilly, Saint-Cloud, France),
VM26: teniposide (Vehem, Sandoz; Rueil-Malmaison, France);
VP16: Etoposide (Vepeside; Sandoz; Rueil-Malmaison, France).

Cells were maintained in contact with the drugs for 2.5 h at 37°C. The drugs were then discarded, the cells were washed with culture medium, and complete culture medium was added (100 μg/well).

The assay method used was basically that described by Volm *et al.* [7]. Tritiated thymidine ([$3H_3$]dThd) was added (52 Ci/mM; final concentration 1.5 μCi/ml) and incubation was continued for 15.5 h. The medium was discarded, and the cells were washed with cold phosphate buffered saline and trypsinized for 5 min at 37°C. As soon as the cells began to detach from the bottom, ice-cold trichloroacetic acid was added to a final concentration of 150 mg/ml and left for 1 h at 0°C.

The DNA content of each discrete well was collected and precipitated on a glass fiber filter using a Skatron cell harvester (Flow Laboratories). After digestion of the precipitated material for 2 h at 37°C using Lumasolve (Lumac), the radioactivity of the filter was counted with a Packard liquid scintillation counter; results were expressed as the percentage of radioactive incorporation relative to a control without the drugs. Each result was the mean of three measurements, and the experiment was repeated three times.

Clonogenic growth in soft agar

Cells were cultured as described by Salmon *et al.* [8]. In brief, cells were suspended on 0.3% agar in enriched Connaught Medical Research Laboratories Medium 1066 (Grand Island Biological Co.) with 15% horse serum (Flow Laboratories, Puteaux, France) in 35 mm Petri dishes containing an underlayer of 0.5% agarose in culture medium. Cells were then incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Plates were examined with an inverted phase microscope 21 days after plating. Positive controls were obtained in the same conditions using a melanoma cell line CAL 1 which gives clones in soft agar [9].

Tumorigenicity

Six-week-old athymic nude mice with the Swiss genetic background (IFFA Credo Laboratories, Lyons, France) were used. For each cell line, 2×10^6 cells in 0.2 ml Ringer lactate solution were injected s.c. in both flanks of 10 mice. Controls consisted of six nude mice injected with CAL 18 A cells (positive controls) or CAL 18 B cells (negative controls) [10].

RESULTS

Morphology under light microscopy

CAL 27. Epithelial cells appeared around the explants 13 days after the initiation of culture. The first passage was performed 1 month later. At the start of culturing, cells contaminated by *Candida albicans* were treated with amphotericin B (Fungizone, Squibb, France) at a dose of 500 μg/100 ml culture medium.

The cell line became established 2 months after the start of culture. Figure 1a shows the cells as seen under a light microscope. These polygonal cells had a highly granular cytoplasm; cell size varied, and growth occurred in a monolayer.

CAL 33. Epithelial cells became visible around the explants 10 days after the start of culture. The fibroblasts which then invaded the culture were eliminated by careful scraping with a rubber policeman. Contamination by *Candida albicans* was treated by amphotericin B. The 1st passage was performed 1 month after the initiation of culture.

Figure 1b shows the CAL 33 cells as they appeared under the light microscope. The nucleus of these even-sized polygonal cells contained one, two or three clearly visible nucleoli. Growth occurred in a single layer.

The two cell lines were maintained until the 40th passage, after which cells were frozen in liquid nitrogen for storage.

Doubling time

Both cell lines grew exponentially up until the 8th day, when they reached a plateau phase. The doubling time was measured during the period of exponential growth: 35 h for CAL 27 and 43 h for CAL 33 (50th passage).

Electron microscopy

The cells of both lines were large (approx. 90 μm) and contained a central nucleus with one or two nucleoli. The cytoplasm featured a granular cyto-

plasmic network associated with numerous free ribosomes. Certain cells had a well-developed Golgi body with pinocytosis vesicles. Mitochondria and lysosomes were visible. Bundles of microfilaments were also seen within the cytoplasm. The cells were joined together by zonula adherens type junctions. The cytoplasmatic membrane occasionally showed slight expansions (Fig.2).

Immunochemical studies

Results were highly positive, both as concerns the original biopsies and the cultures of CAL 27 and CAL 33 on slides (Fig. 3a and 3b); all of the epithelial cells were more or less well marked. The cells thus conserved the characteristics of differentiation specific to keratinocytes even after passage in cell lines.

Chromosome studies

CAL 27. Cytogenetic analysis of cell line CAL 27 revealed moderate hyperploidy; the average number of chromosomes per cell analyzed was 43 (Fig. 4). One of the chromosomes of certain pairs was always (pairs 8,9,11,13,22) or almost always (pairs 12,21) missing. Chromosomes arising from the alteration of the complex structures were visible in all cells; certain of these chromosomes were constant, and thus constitute markers, but none of these elements could be identified with accuracy (Fig. 5).

CAL 33. Analysis of cell line CAL 33 showed moderate hyperploidy; the average number of chromosomes per cell was 49 (Fig. 4). One of the chromosomes of certain pairs was absent (pairs 3,8,9,18,X). Altered chromosomes were also visible in all cells, but they were less numerous and especially less varied than in CAL 27 cells. Seven markers were noted, five of which were identified:

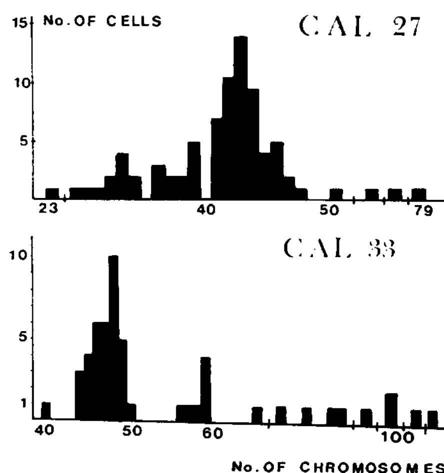

Fig. 4. Chromosome number distribution in a sample of 100 *cells of CAL* 27 *and* 100 *cells of CAL* 33.

3p+, iso (7q), Xp+, 9p+, iso (7p). These marker chromosomes can correspond to the normal chromosomes absent from the genome: 3 absent → marker 3p+; 9 absent → marker 9p+; X absent → marker Xp+. The origin of the alteration was determined for markers iso (7p) and iso (7q): a break in a chromosome 7 at the level of its centromere, with duplication of both arms of the chromosome (Fig. 6).

Chemosensitivity

Figure 7 shows the response of CAL 27 and CAL 33 to the 14 different drugs tested. As the median drug concentration was chosen to match the achievable *in vivo* dose [11], the response was evaluated for this same concentration after the longest interval following drug exposure and removal, i.e. T_3 (96 h). The other relative thymidine incorporation values [for higher or lower drug doses and/or shorter intervals after drug removal (T_0 = 24 h; T_2 = 72 h)] are shown solely to put results in a dynamic perspective.

Inasmuch as we did not have the opportunity to study the *in vivo* chemotherapy response in the two patients who provided the original biopsies for these cell lines, it was impossible to establish a relationship between *in vitro* and *in vivo* chemosensitivity. Nevertheless, the effect of drugs on thymidine incorporation paralleled light microscope observations (*in vitro/in vivo* correlation).

VM26, VP16 and ADM produced marked inhibition of thymidine incorporation in both cell lines; CCNU and CPA were strongly active and ARAC and 5-FU were mildly active only on CAL 33; MTX was strongly active only on CAL 27. Neither VDS, VLB, ACTD or CDP had any inhibitory effect on thymidine incorporation in either cell line. Overall, considering the results for all 14 drugs, CAL 33 (with the exception of MTX) seems more sensitive than CAL 27.

Clonogenic growth in soft agar

No clones were observed for either CAL 27 or CAL 33 20 days after plating; the cells had either undergone lysis or were inert, and showed no growth. The experiment was repeated twice, with the same results. Under the same conditions, CAL 1 gave clones in soft agar 10 days after the initiation of culture.

Tumorigenicity

Both CAL 27 and CAL 33 were tumorigenic in athymic nude mice.

CAL 27. Solid tumors developed 6 weeks after injection in eight of the 20 injection sites. Tumor growth was rapid: 3–5 mm per week. Histological examination of grafted tumors revealed a tumor

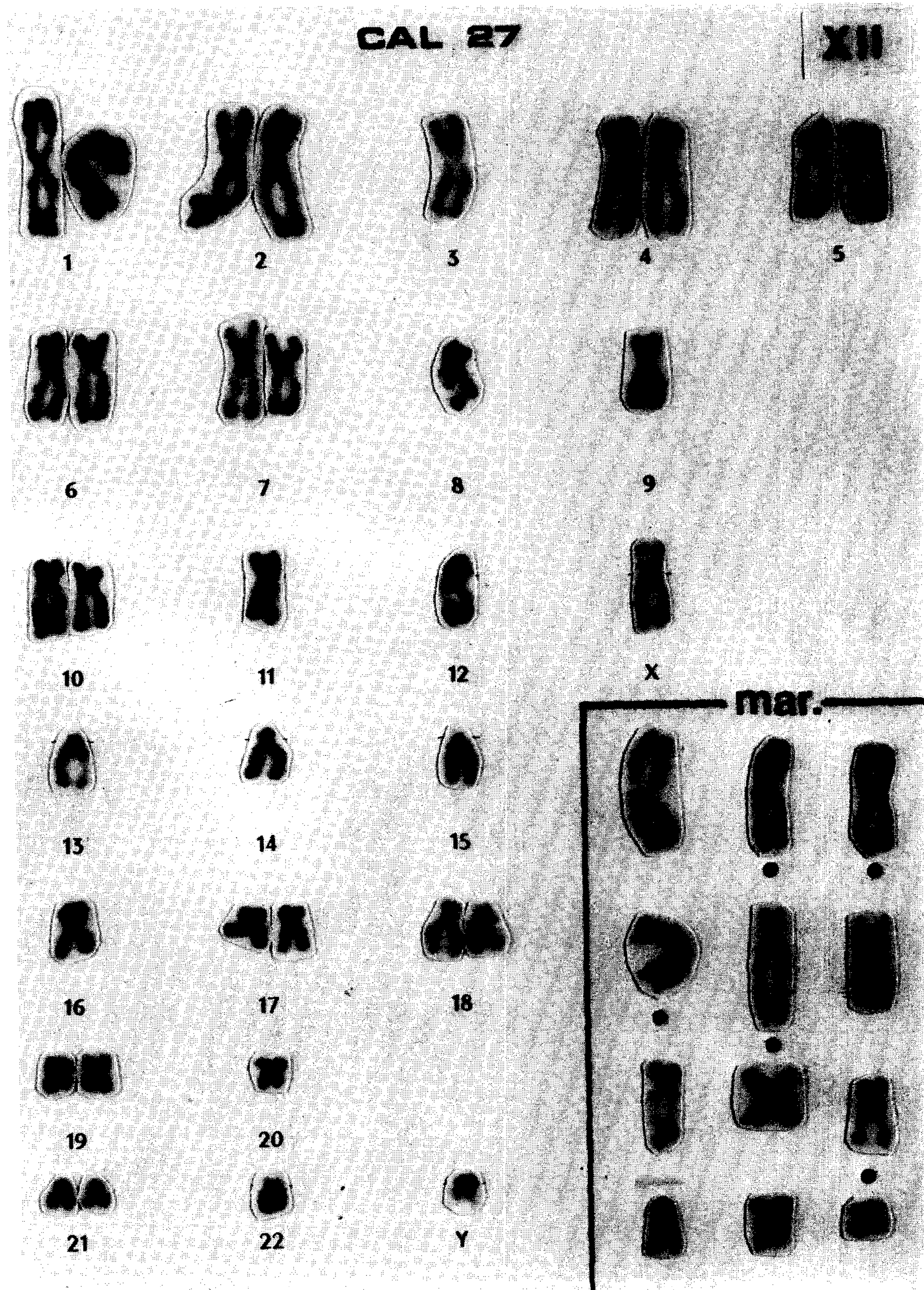

Fig. 5. CAL 27 line karyotype: ● = *constant marker.*

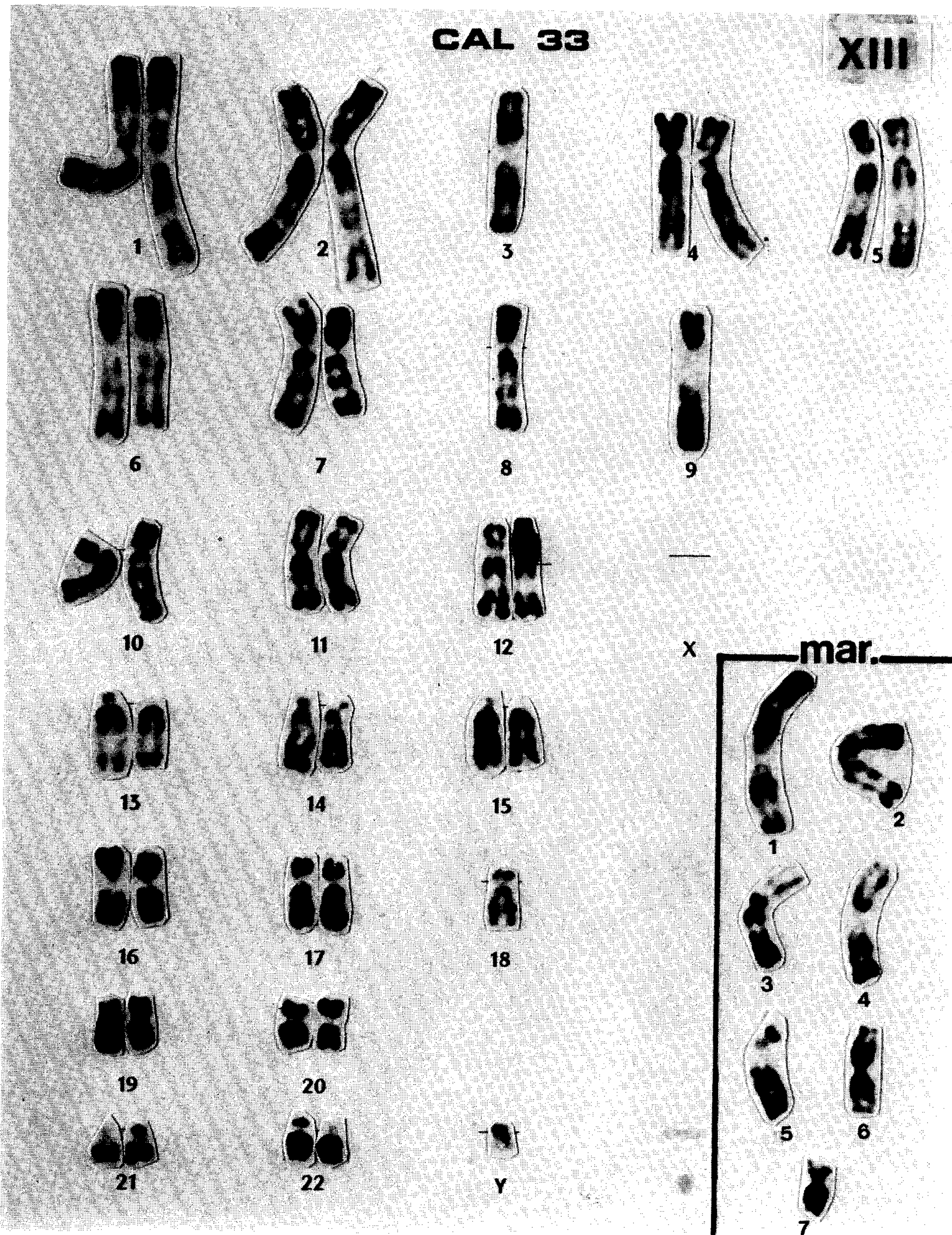

Fig. 6. *CAL* 33 *line karyotype*: *markers*: 1 = 3*p*+, 2 = *iso* (7*q*), 3 = *Xp*+, 4 = 9*p*+, 5 = *der* (9)??, 6 = *iso* (7*p*), 7 = ??.

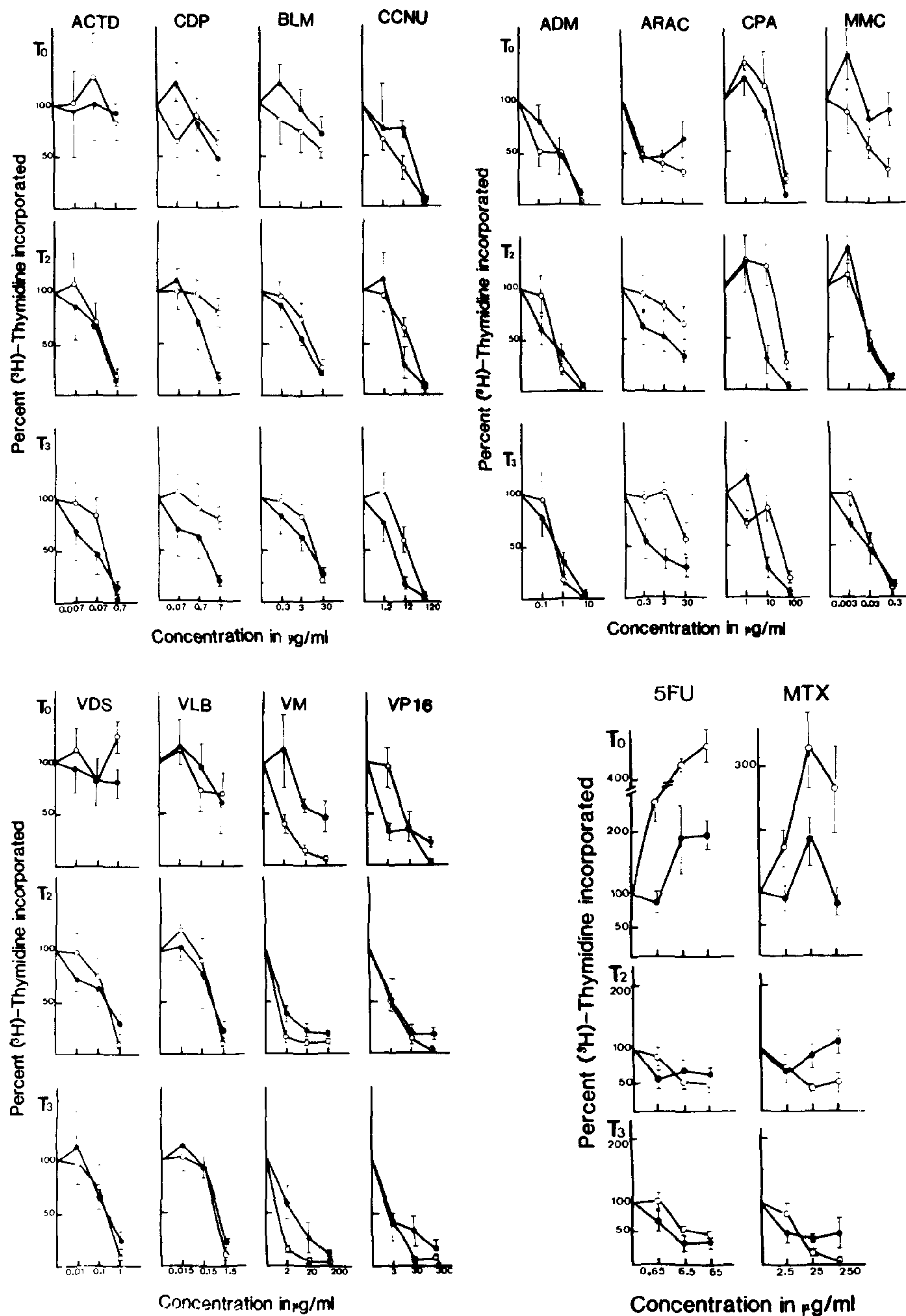

Fig. 7. Effect of 14 *antineoplastic drugs on* [^{3}H]*dThd incorporation* (*abbreviation, drug, concentrations used in* µg/ml). *ADM* = *adriamycin* (0.1,1,10), *ACTD* = *actinomycin D* (0.007,0.07,0.7), *ARAC* = 1-β-D-*arabinofuranosylcytosine* (0.3,3,30), *BLM* = *bleomycin* (0.3,3,30), *CDP* = cis-*platinum* (0.07,0.7,7), *CPA* = *Asta* 7557 (*active cyclophosphamide derivative* (1,10,100), *CCNU* = 1-(2-*chloroethyl*)-3-*cyclohexyl*-1-*nitrosourea* (1.2,12,120), 5-*FU* = 5-*fluorouracil* (0.65,6.5,65), *MMC* = *mitomycin* (0.003,0.03,0.3), *MTX* = *methotrexate* (2.5,25,250), *VDS* = *vindesine* (0.01,0.1,1), *VLB* = *vinblastine* (0.015,0.15,1.5), *VM* = *teniposide VM*-26 (2,20,200), *VP*16 = *etoposide* (3,30,300), ○ *CAL* 27, ● *CAL* 33. *Points* = *percentage of incorporation of untreated cells, bars* = *standard deviation of the mean* (*SDM*).

mass with the characteristics of well differentiated squamous cell carcinoma at varying degrees of maturity.

CAL 33. Solid tumors developed 4 weeks after injection in five of the 20 injection sites. Tumors grew 1–2 mm per week. Histological examination of the grafted tumors revealed that these masses possessed the properties of an invasive, locally keratinizing highly orthoplastic squamous cell carcinoma.

DISCUSSION

Although cell lines CAL 27 and CAL 33 were both obtained from squamous cell carcinomas of the tongue, this study underlines the differences that occur among cancers of the same histological type. This is one of the causes of the difficulties encountered by clinicians with the use of standardized cancer treatments, and has called for development of personalized therapies. These differences are also reflected in the clinical disease course of these cancers in different patients.

The use of culture techniques specific to normal keratinocytes favors the obtainment of SCC lines [4]. However, such techniques can increase the dependence of cells on factors produced by fibroblasts and on anchorage favorable to growth.

Chemosensitivity studies demonstrated that, despite their common mechanism of action as DNA alkylating agents, CDP, CCNU, CPA and MMC did not exhibit any pattern of cross resistance, as previously reported [12].

5-FU and MTX produced a marked increase in thymidine incorporation due to endogenous thymidine pool depletion at T_0 followed by a considerable decrease due to inhibition of DNA synthesis with time (T_2 and T_3). By contrast, the antimetabolite ARAC caused a similar, rapid decrease for both cell lines at T_0 followed by recovery for resistant CAL 27 only and by persistent inhibition for sensitive CAL 33. Further investigations are required to determine whether this is a case of inducible resistance or recovery after potentially lethal damage [13].

Chemosensitivity testing based on thymidine incorporation assays have the advantage of being relatively rapid (4 days) and less time-consuming than more established tests such as the Human Tumor Stem Cell Assay (HTSCA) [8], and has a wider range of applications. In the present case, neither CAL 27 nor CAL 33 was able to grow and thus HTSCA could not be performed.

Observations on tumorigenicity in nude mice are comparable to the disease evolution in the patients who provided the original biopsies. Overall, CAL 33 cells are more differentiated than CAL 27 cells.

This study demonstrates that although it is difficult, tumorigenic squamous cell carcinoma lines can be obtained under standard culture conditions, in other words without any selective constraints. The difficulty of the procedure is reflected by the low rate of success for such cultures, which is only 2% in our experience.

All of our observations indicate that the behavior of these two cell lines in the nude mouse and in culture is similar to that of the original cancers from which they were derived. CAL 27 and CAL 33 thus constitute two good models for *in vitro* cancer studies. In particular, the search for membrane antibodies attached to neoplastic cells provides more interesting results when investigations are conducted on several different cell lines of the same pathologic type.

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REFERENCES

1. Easty DM, Easty GC, Carter RL, Monaghan P, Pittam MR, James T. Five human tumor cell lines derived from a primary squamous carcinoma of the tongue, two subsequent local recurrences, and two nodal metastases. *Br J Cancer* 1981, **44**, 363–370.
2. Krause CJ, Carey TE, Ott RW, Hurbis C, MacClatchey KD, Regesi JA. Human squamous cell carcinoma. *Arch Laryngol* 1981, **107**, 703–710.
3. Moore GE, Merrick SB, Woods LK, Arabasz NM. A human squamous cell carcinoma cell line. *Cancer Res* 1975, **35**, 2684–2688.
4. Rheinwald JG, Beckett MA. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas. *Cancer Res* 1981, **41**, 1657–1663.
5. Pitman SW, Tucker A, Sasaki C. Establishment and characterization of a human squamous head and neck cancer cell line. *Proc Am Assoc Cancer Res* 1981, **24**, 5–10.
6. Dutrillaux B, Lejeune J. Sur une nouvelle technique d'analyse du caryotype humain. *C R Acad Sci Paris* 1971, **272**, 2638–2642.
7. Volm M, Kaufman M, Hinderer H, Goertler J. Schnellmethode zur sensibilitätstestung malignen Tumoren. *Klin Wochenschr* 1970, **48**, 374–376.
8. Salmon SE, Hamburger AW, Soehnlen B, Durie BGM, Alberts DS, Moon TE. Quantitation of differential sensitivity of human tumor stem cells to anticancer drugs. *N Engl J Med* 1978, **298**, 1321–1327.
9. Courdi A, Gioanni J, Lalanne CM *et al.* Establishment, characterization, and response to cytotoxic and radiation treatment of three human melanoma cell lines. *In Vitro* 1983, **19**, 453–461.

10. Gioanni J, Courdi A, Lalanne C *et al.* Establishment, characterization, chemosensitivity, and radiosensitivity of two different cell lines derived from human breast cancer biopsy. *Cancer Res* 1985, **45**, 1246–1258.
11. Alberts DS, Chen MS, Salmon SE. Tabular summary of pharmacokinetic parameters relevant to *in vitro* drug assay. In: Salmon SE, ed. *Cloning of Human Tumor Stem Cells.* A Liss, New York, 1980, 351–359.
12. Frei III E, Cucchi C, Rosowsky A *et al.* Alkylating agent resistance: *in vitro* studies with human cell lines. *Proc Natl Acad Sci USA* 1985, **82**, 2158–2162.
13. Barranco SC, Novak JK, Humphrey RM. Studies on recovery from chemically induced damage in mammalian cells. *Cancer Res* 1975, **35**, 1194–1204.