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Perspectives and Commentaries

Biological Studies of Ten Human Squamous Carcinoma Cell Lines: an Overview

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Abstract—*Ten cell lines established from surgical specimens of human squamous carcinomas of the tongue and larynx have been investigated with respect to their motility, ultrastructure, karyotypes, certain biochemical features, interaction with normal epithelial and stromal elements and capacity to infiltrate three-dimensional organoid systems. All the cell lines have maintained several morphological and biochemical characteristics indicating a common origin, although the extent to which each line displays this heritage is variable. The phenotypes of each of the individual cell lines are, however, notably stable. Data are provided for epithelial surface markers (including epidermal growth factor, EGF) and for the synthesis and release of prostaglandins and proteases which may be involved in invasive mechanisms. Encounters between the cell lines and organoid substrata (embryonic chick heart spheroids, human amnion, chick chorioallantoic membrane) are described: the results indicate a scale of invasiveness ranging from lack of penetration to full-thickness infiltration by cells showing various distinctive growth patterns. Correlation between* in vitro *and* in vivo *findings is discussed, and it is suggested that the biological heterogeneity of the lines may reflect inherent properties of the original carcinoma cell populations which are more distinctly expressed* in vitro.

INTRODUCTION

TECHNIQUES now available to establish cell lines from animal and human tumours provide valuable opportunities to investigate many features of neoplastic cells under controlled *in vitro* conditions. By the late 1970s a considerable body of knowledge had accumulated in relation to the properties and behaviour of normal fibrocytes and their malignant counterparts. It soon became apparent, however, that many of the concepts based on the study of these fibrocyte–sarcoma systems were not directly applicable to normal epithelial cells and to the carcinomas which represent the majority of neoplasms in man. This limitation emphasized the need for a greater availability of adequately characterized carcinoma cell lines derived from human tumours. A number of permanent lines from human carcinomas have now been established including several squamous cancers of the head and neck (for references see [1]). These lines have been used for various purposes including the development of assays for cytotoxic drugs and, in particular, for investigating keratinocyte differentiation in neoplastic cells. Our own efforts have been directed mainly towards the analysis of certain behavioural aspects of squamous cells in the context of invasiveness. For this work we have used a series of 10 cell lines derived from human carcinomas of the tongue and larynx. The potential of these lines to stimulate

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and facilitate work on squamous carcinomas was recognized by various research groups, and a number of separate studies were set up which reflected the specific and often diverse interests of the investigators involved. Coordination of this work was not envisaged at first and only realized (to a limited extent) in the last few years. We cannot present a complete account of all the properties studied in all the cell lines and we are aware of gaps such as the lack of data on proliferation kinetics; a considerable amount of information has, however, accumulated on the behaviour of squamous cell carcinomas of the head and neck region in a variety of experimental systems.

ORIGINS OF CELL LINES: CLINICOPATHOLOGICAL DATA

Six of the cell lines used in these studies were established by Easty *et al.* [1] in London and are designated as LICR (LON) HN1 to HN6. Another four lines were developed by H. Felix in Zürich and are designated as ENTZ-HTC 1 and 2, and ENTZ-HLC 1 and 2. Origin and relevant pathological features are summarised in Table 1. The cell lines were investigated with regard to the properties listed in Table 2.

MORPHOLOGY AND STUDIES ON MOTILITY

The morphology of the cells was determined by

Table 1. Origin of cell lines and clinicopathological data

Code	Age, sex	Anatomical site	Preoperative CT/RT	Histological differentiation of primary tumour	Metastases in regional lymph nodes	Subsequent survival
LICR (Lon) HN-1	51, M	Tongue	CT, RT	Moderate to poor	0/31	Dead at 8 months
-2	49, F	Larynx	RT	Poor	0/54	Alive at 5 yr
-3	65, M	Tongue	CT, RT	Well to moderate	2/17	Dead at 13 months
-4	57, M	Larynx	CT, RT	Moderate to poor	–	Dead at 14 months
-5	73, M	Tongue	CT, RT	Well to moderate	4/28	Dead at 4 months
-6	54, M	Tongue	CT, RT	Well to moderate	11/46	Dead at 3 yr
ENT2 – HTC 1		Tongue	No	Well		
2		Tongue	No	Well		
HLC 1		Larynx	No	Well to moderate		
2		Larynx	No	Well		

Table 2. Biological parameters studied and lines used

	Oral carcinomas						Larynx carcinomas			
	HN-1	HN-3	HN-5	HN-6	HTC-1	HTC-2	HN-2	HN-4	HLC-1	HLC-2
Clinical pathology	+	+	+	+	+	+	+	+	+	+
Behaviour *in vitro* on glass	+	+	+	+	+	+	+	+	+	+
Cell morphology	+	+	+	+	+	+	+	+	+	+
Karyotype	+	+	+	+	+	+	+	+	+	+
Biochemical properties										
– Cytokeratins	+	+	+	+	nd	nd	+	+	nd	nd
– CEA, EMA, EGF	+	+	+	+	nd	nd	+	+	nd	nd
– β-HCG	+	+	+	+	nd	nd	+	+	nd	nd
– Proteinases	+	+	+	+	nd	+	+	+	nd	nd
– Ca^{2+} binding proteins	+	nd	nd	+	nd	nd	+	nd	nd	nd
Interaction with other cells and tissues										
– Normal epithelial cells	+	nd	+	+	nd	nd	+	nd	nd	nd
– Cartilage	+	nd	nd	nd	nd	nd	nd	+	nd	nd
– Bone	+	+	+	+	nd	nd	+	+	nd	nd
– Human amnion	nd	+	nd	nd	nd	+	+	nd	+	+
– Embryonic chick heart	+	+	+	+	nd	nd	+	+	nd	nd
Behavior in animal hosts										
– On the CAM	+	+	+	+	nd	+	+	+	nd	nd
– In nude/immuno-suppressed mice	+	+	+	+	+	+	+	+	+	+

nd = not done

means of phase contrast, transmission (TEM) and scanning electron microscopy (SEM). Motility on glass substrates was investigated with time-lapse cinematography.

Phase contrast photography and time-lapse cinematography

Cells from all 10 lines form epithelioid islands, ranging from loose aggregates (HN-1 and HTC-2) to strongly cohesive clumps (HN-2). The cohesion of cells reflected the degree of motility within and around the islands which varied considerably for the different lines. Translocative motility, locomotion, was most obvious in lines which formed islands with active cells along the borders. These peripherally placed cells frequently detached, migrated, and reattached either to single cells which they met on the way, or to cells of neighbouring islands. The lines which formed loose aggregates (HN-1, 5, and HTC-2) showed the least degree of translocative motility and exhibited what we have termed 'stationary' motility — that is, motility without translocation of the entire cell [2]. The motile behaviour of lines HN-2 and 6 on glass is illustrated in Figs. 1a and b, 2a and b. Activity within the islands also occurred, ranging from changes of shape (stationary motility) to actual positional shifts. Single cells sometimes migrated over the surface of islands [2].

Cells from one of the lines (HN-1) were implanted into the peritoneal cavity of nude mice. The tumour cells invaded the mesentery, and time-lapse films revealed that the cells displayed the same stationary type of motility as on glass [3].

Time-lapse cinematography performed on populations of normal cells from human buccal mucosa and pharynx showed that they, too, were motile on glass surfaces. The mode of translocation was identical to that of cancer cells but detachment of single cells from the edge of the islands was rather infrequent, being comparable to that seen in lines HN-1, 2 and 5 (Haemmerli, unpublished data).

The initial microcinematographic studies were later extended to include investigation of the cytoskeleton, particularly the microfilaments known to be involved in motility and adhesion to substrates. Time-lapse cinematography, reflection contrast microscopy, and TEM of horizontal sections were carried out on the same cells from lines HN-1 and 6. Similar cells from these two lines were stained by immunofluorescence for actin, tubulin, vimentin and prekeratin [4]. In general, it was found that the configuration of actin filaments was different in cells fixed during translocation and in cells engaged in non-translocative activities — a mesh-like pattern was demonstrated in the former, compared with a compact arrangement in bundles in the latter. No difference in localisation was seen for microtubules and 10 nm filaments. Both cell lines contained abundant intermediate filaments of the vimentin type, but HN-1 cells lacked demonstrable prekeratin.

The influence of the promoting agent TPA (12-0 tetradecanoylphorbol-13-acetate) on the speed of movement of HN-2 cells was studied by Marcel and Bruynel (unpublished data). Time-lapse films showed that addition of 10 or 50 ng/ml TPA speeded up the cells by a factor of between 2 and 6 over periods of 4–8 hr. The differences between velocities before and after addition of TPA were highly significant.

Transmission electron microscopy (TEM)

The ultrastructure of the HN lines was first described in 1981 [1] as showing the two unequivocal characteristics of squamous epithelium: desmosomes and tonofilaments. One line — HN-1 — formed multilayers under *in vitro* conditions with a thickening of parts of adjacent cell membranes but without completely formed desmosomes. Comparative ultrastructural studies on lines HN-1, 2 and 5, using primary cell cultures and cells from higher passages (20–30 and more), revealed that the characteristic features of these lines had not changed with time (Felix, unpublished data). HN-1 cells still lacked well-developed desmosomes; HN-2 cells contained abundant rough endoplasmic reticulum; HN-5 cells displayed large amounts of bundled tonofilaments and many well-developed desmosomes. HN-5 cells tend to develop blebbing protrusions *in vitro*, and it was shown that these regions contained various organelles such as rough endoplasmic reticulum and mitochondria. Some of these ultra-structural findings are shown in Figs. 3–8.

The well-differentiated HN-5 cell line has been used as a model to compare keratinocyte differentiation in squamous carcinomas and in normal squamous epithelium [5, 6]. The ultrastructural features of keratin formation are strikingly similar in the two cell types. HN-5 cells, grown on collagen gels, formed organized multilayered structures with identifiable basal and spinous cells, keratohyalin granules and a basal lamina at the stroma-epithelial interface.

Scanning Electron Microscopy (SEM)

The surface architecture of the lines visualized by SEM revealed features which range from smooth to villous to blebby structures. Two lines displayed a certain regularity in the expression of surface structures: HTC-1 exhibited a particularly dense arrangement of microridges, and HN-5 cells displayed blebs and long microvilli as a relatively stable property of individual cells (see previous section). The relationship between surface architecture and cellular shape and motility has been discussed in previous communications [7, 8].

Examples of the various surface features are shown in Figs. 9–14.

Motility and general morphology, including TEM and SEM aspects, have been checked at varying time intervals during the past 4 yr. The characteristic features of the different lines remained unchanged.

KARYOTYPIC ANALYSIS

Preliminary studies by Butler (see 1) with lines HN-1 to HN-4 established that all the cells were in the triploid region, all four lines contained distinctive marker chromosomes, and there was no evidence of contamination between the cell lines or from non-human cells. More detailed investigations have now been made with higher passages of lines HN-1 to 6, a subline of HN-6 derived from a lymph node metastasis (HN-6n) and lines HLC-1, HTC-1 and 2 [9]. Three different methods of chromosome staining were used: G-banding, to distinguish between normal and marker chromosomes; C-banding, to determine if the centromeric region was involved in translocations; and silver staining to visualize the nucleolus-organizing region (NOR), the size of which is directly related to protein production. It was found that the lines had retained their tendency towards triploidy, with some cells reaching into the tetraploid area. All of them contained marker chromosomes. Four of the six HN lines, established from tissues which had been exposed to preoperative irradiation and/or chemotherapy, contained a greater number of chromosomes per cell than the lines derived from patients who were not pretreated (HTC-1, 2 and HLC-1). A constant chromosome aberration, involving chromosome no. 1, was seen in all cell lines. One marker chromosome (M3) was unique to the HN lines. Whether the presence of this chromosome was a consequence of the preoperative chemotherapy and/or irradiation is unclear. The localization and amount of nucleolus-organizing regions (NORs) varied among the different tumour lines. NOR-bearing chromosomes were especially evident in marker chromosomes and occurred in lines from four tongue carcinomas (HN-1, 3, 5, HTC-1) and from one laryngeal carcinoma (HLC-1). Further NORs were concentrated on single chromosomes as well as in isolated spots, and appeared approx. 10–30 times larger than the total amount of NOR in the normal human genome.

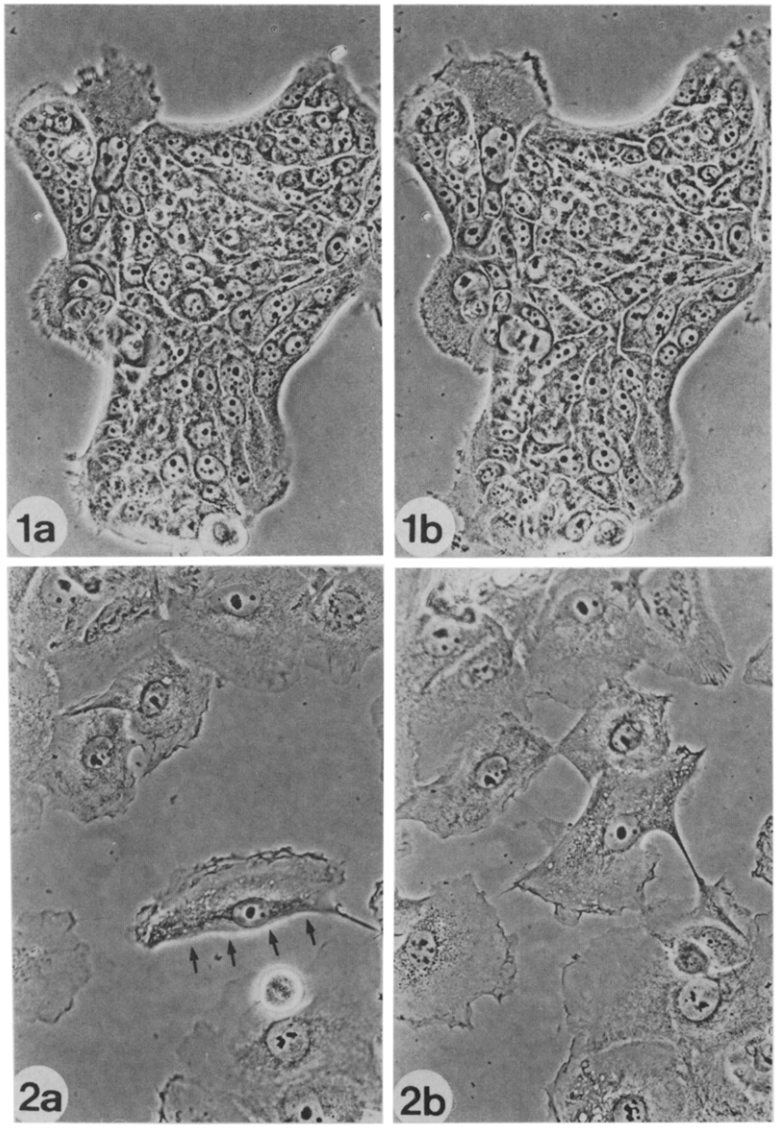
BIOCHEMICAL PROPERTIES

Detailed analyses of *specific cytokeratins* in cell lines HN-1 to 6 using SDS–PAGE electrophoresis were made by Knight [10] and Knight *et al.* [11]. Cytokeratins could not be identified in HN-1 cells, while cells from lines HN-2, 3, 4 and 6 exhibited variable amounts of predominantly low mol. wt cytokeratins. One finding of particular interest was the similarity of cytokeratin expression in the tongue carcinoma line HN-5, which contains large amounts of tonofilaments, and in normal keratinocytes grown *in vitro*. The close similarity of the ultrastructural features of keratin formation in this cell line and in normal keratinocytes has already been noted. A single clone of the HN-5 line, designated B2A(4), expressed the same degree of differentiation as the parent HN-5 cells.

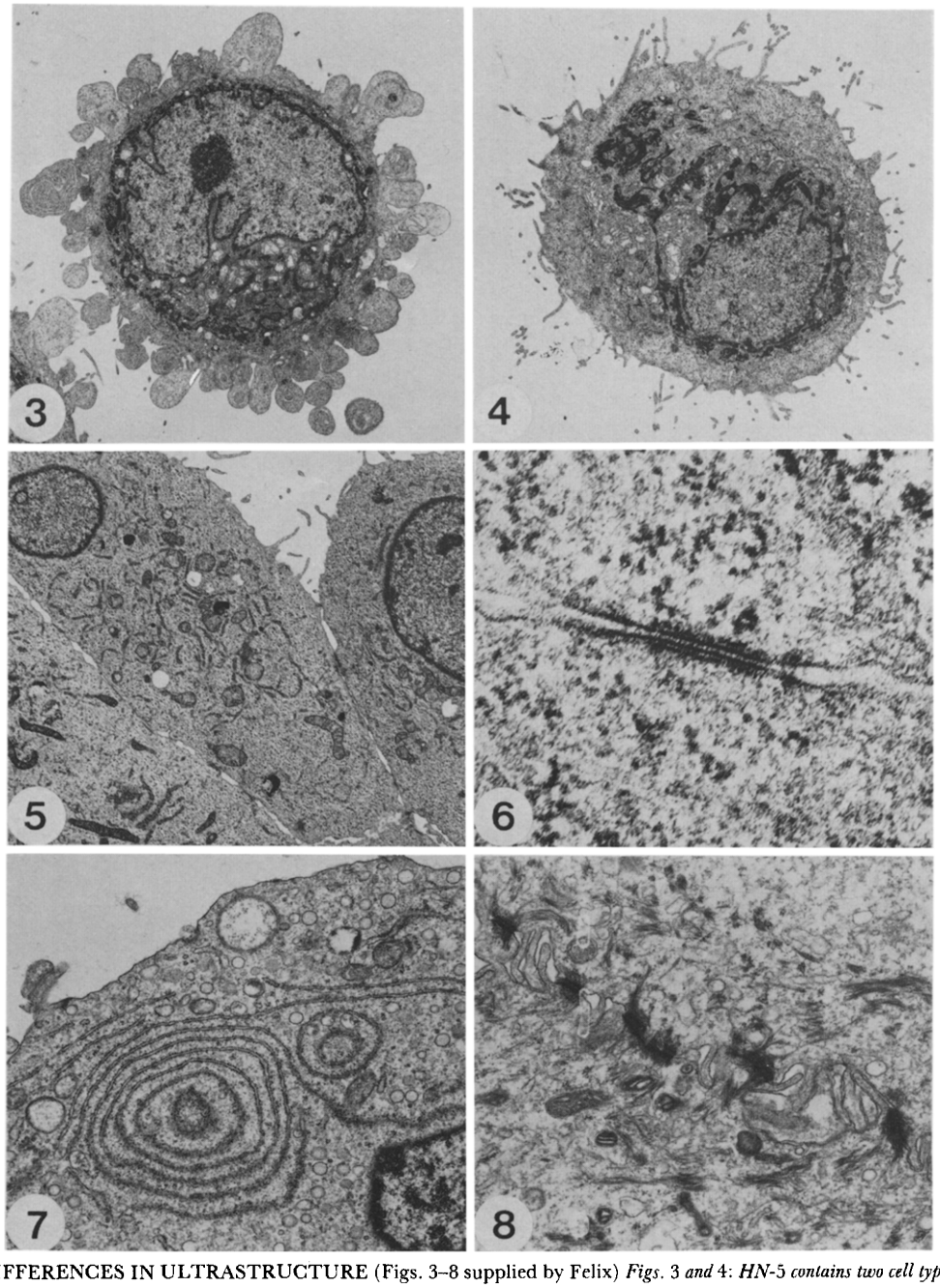
Carcinoembryonic antigen (CEA), a membrane component of several epithelial cell types, was released in significant amounts by lines HN-1, 2, 3 and 4. Biochemical and immunocytochemical investigations with peroxidase-labelled polyclonal antibodies demonstrated both CEA and epithelial membrane antigen (EMA) on the surface of cells from all HN lines [12]. The quantities of antigen expressed were found to vary significantly between different cells in any single culture (Ormerod *et al.*, in preparation). EMA and CEA were distributed as patches on the surfaces of unfixed cells in contrast to the even distribution of the two antigens on the cell surface of breast cancers [13]. These observations may point to a difference in the interaction between contiguous cell surfaces, reflecting in turn the different origin of the cell lines. Further investigations of the distribution of EMA on the surfaces of the squamous cell lines revealed that glutaraldehyde fixation converted the patchy distribution of EMA to an even one and doubled the number of antibody-binding sites. There was no evidence that unfixed cells took up antigen-antibody complexes by endocytosis. It was suggested that the patchy distribution of EMA on unfixed cells was not induced by antibody, and that glutaraldehyde caused a surface rearrangement which both increased the detectable quantities of surface antigen and spread it more evenly over the cell surface (Figs. 15, 16).

Small but significant quantities of *immunoreactive beta-human chorionic gonadotrophin* (beta-HCG) were produced by all lines except HN-4. Ellison [14] established that, somewhat unusually, the production of beta-HCG by these cell lines was higher when the cells were in the early growth phase than when cell proliferation had decreased after the cultures had reached confluence. Ectopic beta-HCG produced by 8 of the cell lines has recently been shown to be identical in its gel exclusion and HPLC properties to placental beta-HCG and, in radioimmunoassay, it yields a dilution curve parallel to that of placental beta-HCG [15].

High levels of *epithelial growth factor* (EGF) were detected on the surface of HN-5 cells by Ellison (personal communication), and this observation has now been extended [16–18]. EGF receptor levels were measured in eight of the squamous carcinoma cell lines and in normal and SV-40-

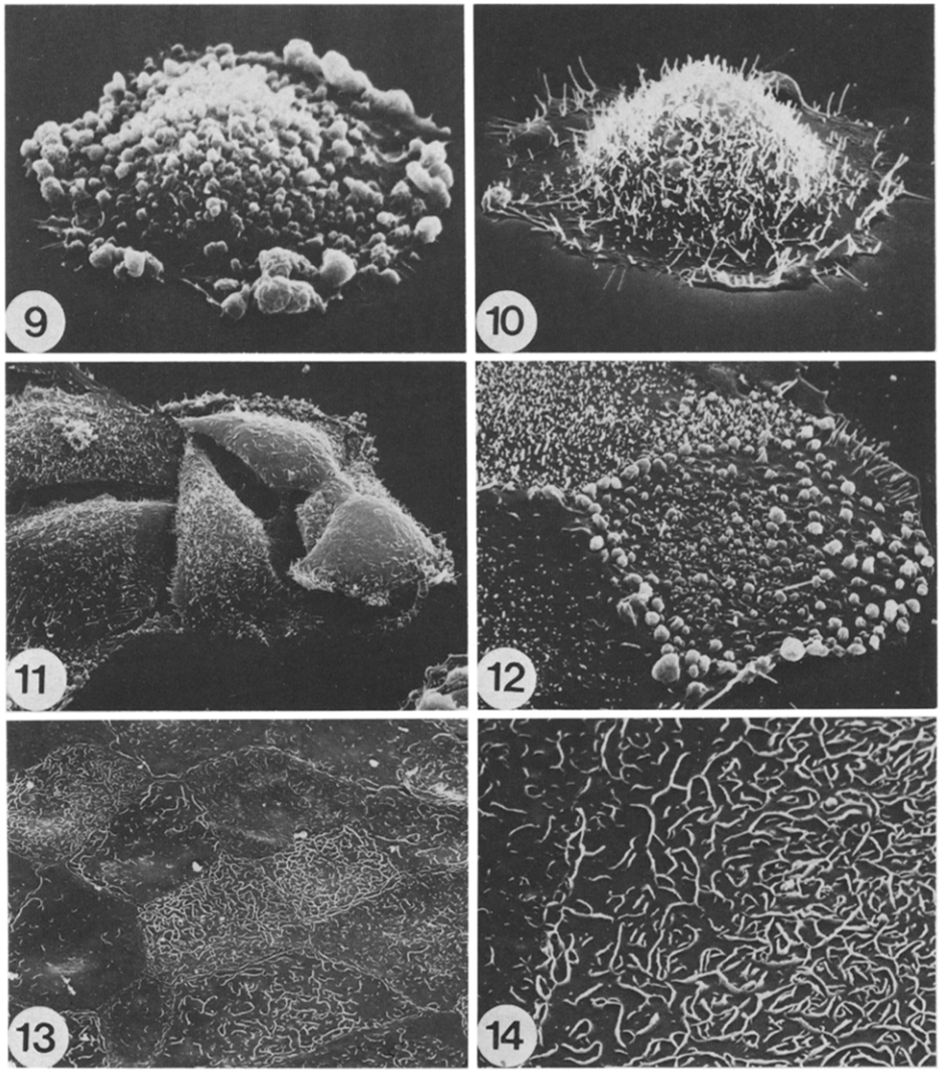


MOTILITY (Figs 1a, b and 2a, b supplied by Haemmerli) *Figs.* 1a, b. *Cohesive island formed by HN-2 cells with little activity of border cells. Time interval:* 2 hr. *Figs.* 2a, b. *Very active population (HN-6) with locomotory cells and rapid rearrangement of cellular positions. A translocative cell is indicated by arrows. Time interval*: 2 hr. *Time-lapse film, phase contrast* × 200 (*Figs.* 1a, b), × 250 (*Figs.* 2a, b).



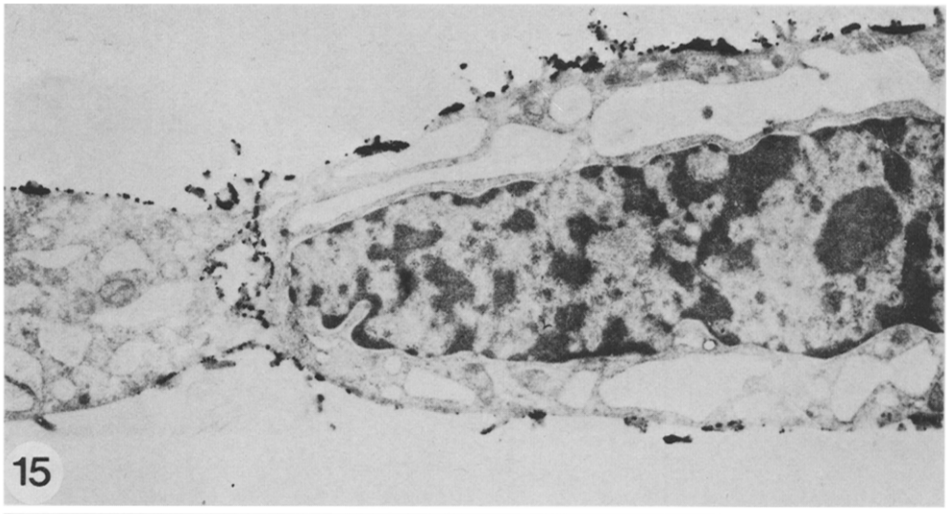
DIFFERENCES IN ULTRASTRUCTURE (Figs. 3–8 supplied by Felix) *Figs.* 3 *and* 4: *HN-5 contains two cell types, one with blebs* (*Fig.* 3), *one with long microvilli* (*Fig.* 4). *The blebs possess rough endoplasmic reticulum and mitochondria. Both types have large amounts of bundled tonofilaments.* TEM × 3300

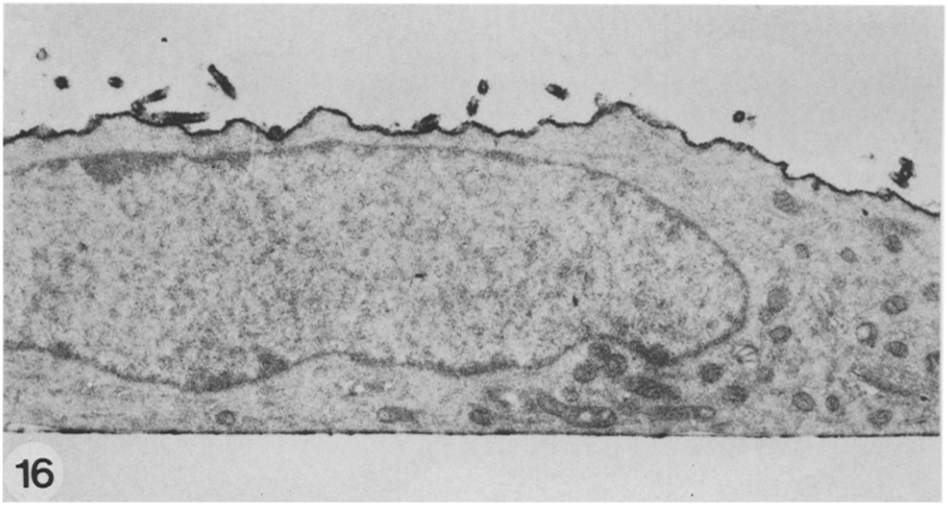
Figs. 5 *and* 6: *HN-1 cells lack tonofilaments and form incomplete desmosomes only. TEM* × 3500 (*Fig.* 5), × 74,000 (*Fig.* 6).
Fig. 7. *HN-2 cells contain large amounts of rough endoplasmic reticulum, frequently arranged in circular arrays. TEM* × 11000.
Fig. 8. *Cells from HLC-2 form cohesive islands with frequent interdigitations and many desmosomes. TEM* × 1300



SURFACE FEATURES (Figs. 9–14 supplied by Felix) *Figs.* 9, 10. *Two HN-5 cells, one blebby (Fig.* 9), *one with long microvilli (Fig.* 10). *SEM* × 1700 (*Fig.* 9), × 1500 (*Fig.* 10).

Figs. 11–14. *Illustrate the variability in surface architecture of the different lines: HN-1 (Fig.* 11), *HN-4 (Fig.* 12), *HTC-1 (Figs.* 13, 14). *SEM* × 800 (*Figs.* 11, 12), × 550 (*Fig.* 13), × 1700 (*Fig.* 14).





IMMUNOPEROXIDASE STAINING FOR CEA AND EMA (Figs. 15, 16 supplied by Ormerod) *Fig.* 15. *Staining for CEA of an unfixed HN-2 cell. Note the patches of staining on the cell surface and the staining of the contiguous cell surfaces. Fig.* 16. *Staining for EMA of an HN-1 cell fixed with* 0.2% *glutaraldehyde in PBS. Note the even stain across the cell surface. TEM* × 10000 (*Fig.* 15), × 7500 (*Fig.* 16).

transformed human keratinocytes. High levels of EGF receptor — 5–50 times normal — were found on the squamous carcinoma cells: HN-5 contained 1.5×10^7 receptors/cell, the highest concentration yet recorded. High levels were also observed in fresh tumours, and it has been argued that the raised EGF concentrations are an important phenotypic component of squamous carcinomas, irrespective of their site of origin.

Three of the cell lines, HN-1, 2 and 6, were investigated for various CA^{2+}-*binding proteins* [19]. All of them contained a distinctive CA^{2+}-binding protein (mol. wt 12000, iso-electric point 4.8, and 2 Ca^{2+} bound per molecule) which was absent from comparable normal tissues. This protein is immunologically and structurally related to parvalbumin and was often found to be associated with (as yet undefined) filamentous structures in the three cell lines. There is evidence that parvalbumin regulates the relaxation process in muscle [20, 21], and it is possible that the parvalbumin-like protein described here may be associated in some way with the motility of these carcinoma cells.

High concentrations of oncomodulin (mol. wt 11500, isoelectric point 3.9) were found in HN-6 cells and low concentrations in HN-2 cells; none was demonstrated in HN-1 cells. These observations confirm an earlier study which showed that oncomodulin was present in only a proportion of the tumours investigated [22]. Clearly, oncomodulin cannot be regarded as a general tumour marker. The S-100 proteins, a family of Ca^{2+}-binding patterns which are preferentially (but not exclusively) present in nerve and fat cells have been detected in primary tumours of the nervous system in man [23] and also in cell lines derived from human melanomas [24]. They have been demonstrated in squamous carcinoma cells from lines HN-2 and 6, but not in HN-1. The function of the S-100 proteins is unknown. Calmodulin, a ubiquitous multifunctional Ca^{2+}-binding receptor which is present in all eukaryotic cells, is thought to contribute to the higher proliferation rate of tumour cells. It was found in notably large quantities in cells from line HN-6, and in lower amounts in lines HN-1 and 2. Although all these Ca^{2+}-binding proteins are structurally homologous and belong to the troponine-superfamily, further studies are needed to identify their functional roles in carcinoma cells of all types.

The capacity of tumour cell lines to destroy tissue proteins has attracted considerable interest. Baici [25] investigated the production and release of various *proteinases* by six of the HN lines and by the HTC-2 line. The HN lines differed from each other in their capacity to produce and release collagenase, elastase, plasminogen activators and cathepsin B-like enzymes. HN-3 and 5 were the only lines to secrete low but significant quantities of active collagenase. The larynx carcinoma line HN-4 appeared to be the most active in producing a cathepsin B-like enzyme. The histochemical and immunocytochemical localization of this enzyme in monolayer cultures of all HN lines was studied by Graf (personal communication). Appreciable amounts of cathepsin B were demonstrated in cells from the tongue carcinoma lines HN-1 and 6 and from the larynx carcinoma HN-4, but only trace quantities were found in the three carcinoma lines HN-2, 3 and 5.

Independent investigations of Type I collagenase activity in the squamous carcinoma cell lines were recently reported by Burman and Carter [26]. Total collagenase levels were significantly increased in only 4 of the 20 tumour cell lines examined but, paradoxically, they were substantially raised in all of six control (non-neoplastic) fibroblastoid lines. Co-cultivation experiments with tumour cells and fibroblastoid cells at a ratio of 1 : 10 provided evidence of enhanced, synergistic collagenolysis. Parallel experiments with supernatant media from the carcinoma and fibroblastoid lines show no enhancement, indicating that intact cells were required for synergism to take place.

Cells from all HN lines released varying quantities of plasminogen activator, the largest amount being produced by HN-4. The liberation of plasminogen activator by this cell line was investigated by Cederholm-Williams (unpublished data) who found two types of enzyme. The first, which constituted some 25% of the total activity, resembled urokinase. It was fibrin-independent and had a mol. wt of 54,000. The second enzyme, responsible for the remaining 75% of activity, was fibrin-dependent and had a molecular weight of 70,000.

INTERACTION BETWEEN TUMOUR CELLS AND STROMAL ELEMENTS: BONE AND CARTILAGE

Interactions between bone and squamous carcinomas of the head and neck were investigated by Tsao [27–29] and Carter [30, 31]. Freshly excised tumours stimulated the release of ^{45}calcium from isotopically-labelled baby mouse calvaria *in vitro* by activating local osteoclasts — thereby reproducing the osteoclastic response which is a consistent feature of bone destruction by squamous carcinomas in surgical material. Bone resorption was regularly but incompletely blocked by indomethacin, suggesting that a mixture of both prostaglandin (PG) and non-prostaglandin factors was produced. It was subsequently shown that most, but certainly not all, osteolytic activity by fresh tumour tissue was attributable to PGE_2. The synthesis and release of calcium-resorbing prostaglandins is not, however, a specific attribute of tumours; control, non-neoplastic tissues regularly showed similar (albeit usually less marked) activities. Subsequent

examination of osteolysis by eleven established squamous carcinoma cell lines (including HN-1 to HN-6), free of any contaminating non-malignant cells, showed that many of the lines released osteolytic factors into the culture medium; activity was not, however, significantly reduced by adding inhibitors of prostaglandin synthesis and the levels of PGE_2 in the culture medium were too low to account for the osteolysis observed *in vitro*.

When cells from HN-5 and HN-6Rt (a line derived from a locally recurrent lesion of HN-6) were grafted subcutaneously into immunosuppressed mice, the animals became increasingly hypercalcaemic as the tumours grew. Administration of indomethacin did not reduce the hypercalcaemia and did not significantly decrease the osteolytic effect of the freshly excised xenograft measured *in vitro* [28]. The evidence available thus indicates that most of the cell lines possess *in vitro* osteolytic activity which is not related to the production of prostaglandins. In addition, two of the lines grown as xenografts in mice produce hypercalcaemia, again attributable to non-prostaglandin-like factors. The chemical identity of the non-prostaglandin factor(s) involved is as yet unknown.

The relative resistance of cartilage to invasion by malignant cells has been well documented both *in vivo* [32] and *in vitro* [33–35]. Morphological studies on squamous carcinomas invading the osseocartilagious framework of the larynx indicated that ossified cartilage was more often infiltrated than normal cartilage, though the resistance of the latter was not absolute [36–38]. In 1981, Pauli *et al.* [39] reported that a low mol. wt fraction, which they named cartilage-derived anti-invasive factor, was responsible for this resistance. The factor was salt-extractable, and cartilage from which it had been removed lost its apparent capacity to withstand tumour invasion. Bracke *et al.* [40] attempted to repeat these observations. They used bovine, avian and human cartilage from various tissue sources, and applied mouse and human tumour cells, inluding those of the line HN-4, to normal and salt-extracted cartilage in the form of monolayer fragments, cell suspensions and aggregates. Human embryonic lung cells and chick embryonic heart cells were used as non-neoplastic control tissues. The confrontation cultures were examined by light and electron microscopy after periods of 1–14 days. Bracke and co-workers could not demonstrate invasion of malignant cells into the matrix of either normal or salt-extracted cartilage, nor did they observe any breakdown of collagen in these cultures. Both normal and malignant cells were, however, found to have occupied pre-existing spaces in the cartilage matrix. Independently, Easty and Easty (unpublished data) used bovine cartilage from the metacarpo-phalangeal joint, extracted in the same manner as described by Kuettner [35] and Bracke [40], and applied suspensions of HN-1 and HN-4 cells. After two weeks, light microscopic studies showed no clear invasion by tumour cells. It thus appears that salt-extractable factors are not solely responsible for the inhibition of invasion into cartilage.

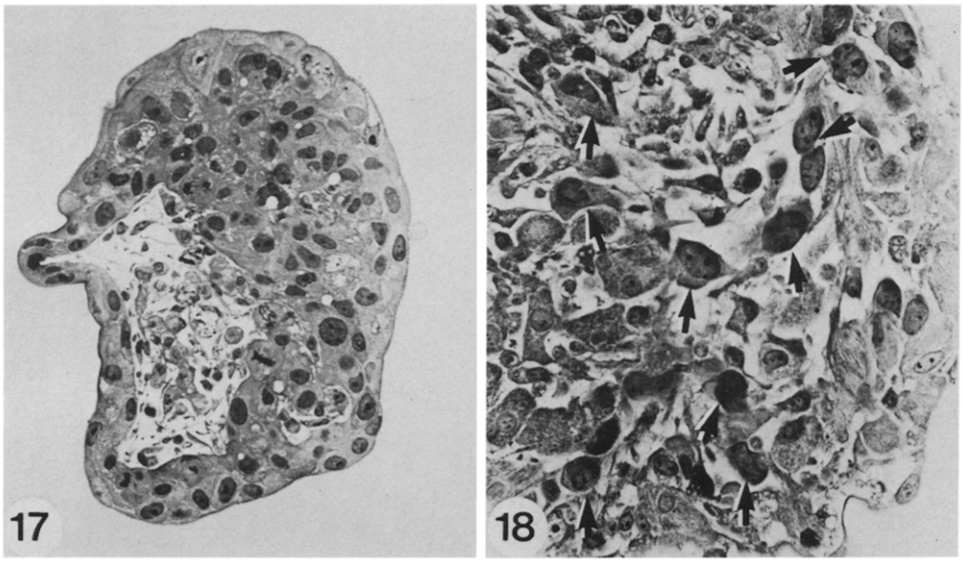
INTERACTION BETWEEN TUMOUR CELLS AND NORMAL EPITHELIAL CELLS IN MONOLAYER CULTURES

One of the main areas of investigation in which we have used the squamous carcinoma cell lines is the interaction between normal and malignant cells under *in vitro* and *in vivo* conditions. Easty *et al.* [41] enclosed circular islands of normal epithelium, cells from lines HN-1, 2 and 6, and fibroblasts with a ring of normal human epithelium leaving an empty space between the two populations.

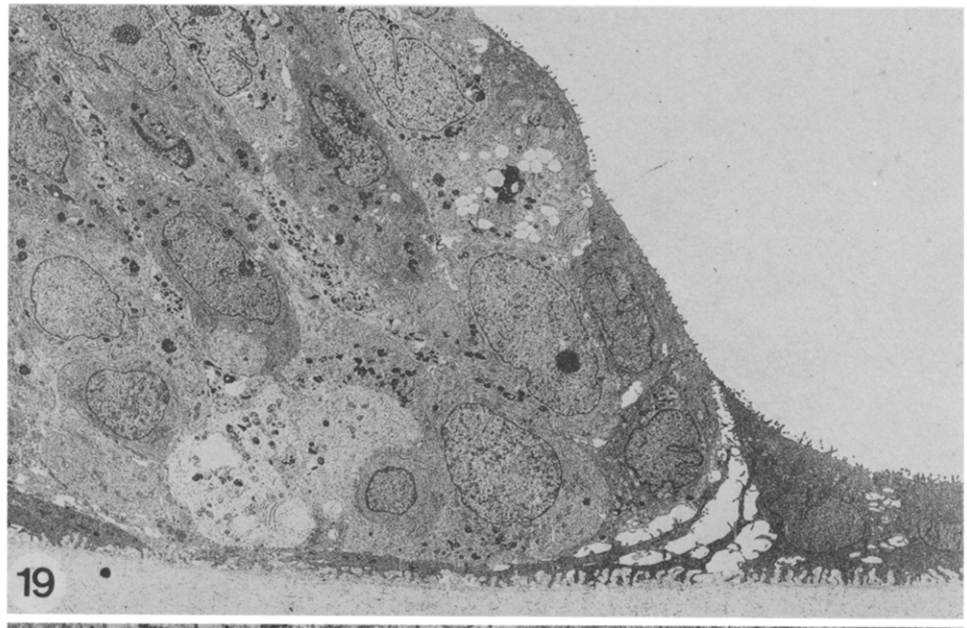
Time-lapse cinematography and reflection-contrast and dark field microscopy were used to study the changes which occurred as the two different populations interacted. The rings of normal epithelium, surrounding cells of the same origin or fibroblasts, started to spread in both directions — outwards and inwards — until they made contact with the centrally placed normal cells. In contrast, if normal epithelium was placed round cells from three tumour lines, spreading and translocation occurred only in an outward, centrifugal direction. Although the cells from all three tumour lines divided and migrated towards and made localized contact with the encircling epithelium, cell-free spaces between the two populations were always maintained. These findings indicate that the outward migration of the normal cells was not induced by proliferation of the carcinoma cells nor by any immediate cell–cell contact. Cells from HN-1 and 6 moved as single cells towards the epithelium, while those from HN-2 advanced as a confluent sheet. Time-lapse films made under phase contrast and reflection contrast illumination also revealed that single cells from HN-1 and 6 freely migrated under and over normal cells from buccal mucosa.

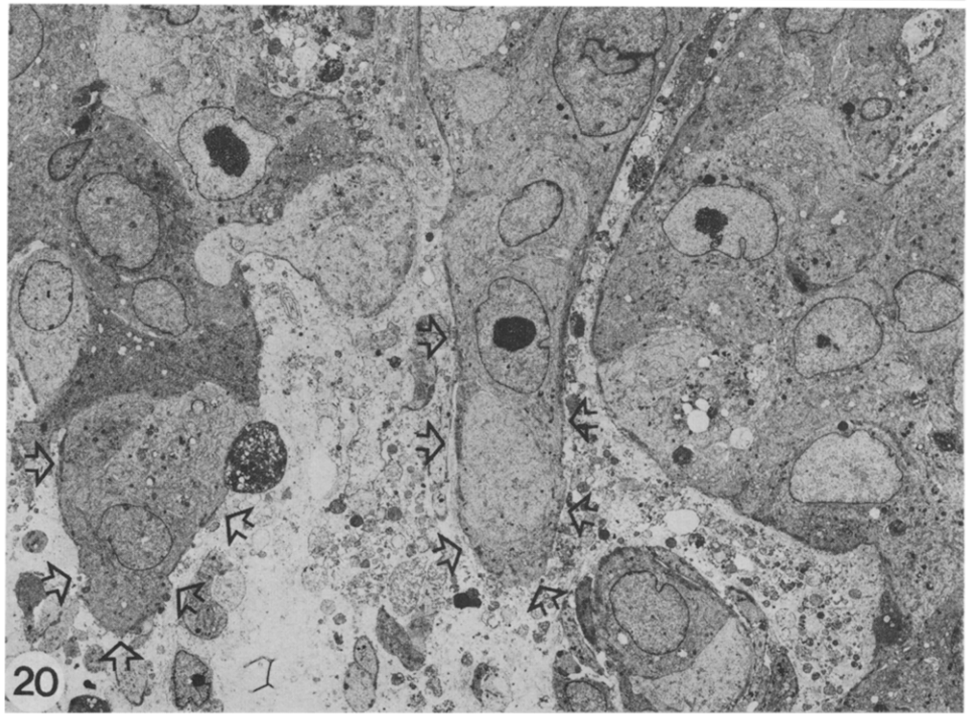
INTERACTION BETWEEN TUMOUR CELLS AND NORMAL CELLS IN THREE-DIMENSIONAL SYSTEMS

Investigations were made with an *organ culture model* in which precultured fragments of cardiac muscle from 9-day chick embryos were confronted with aggregates of tumour cells [42]. The invasive behaviour of the six HN lines was studied, and some results with HN-1 and 4 cells are illustrated in Figs. 17 and 18; see also Marcel *et al.* [43]. It was found that HN-1 cells invaded most rapidly, being

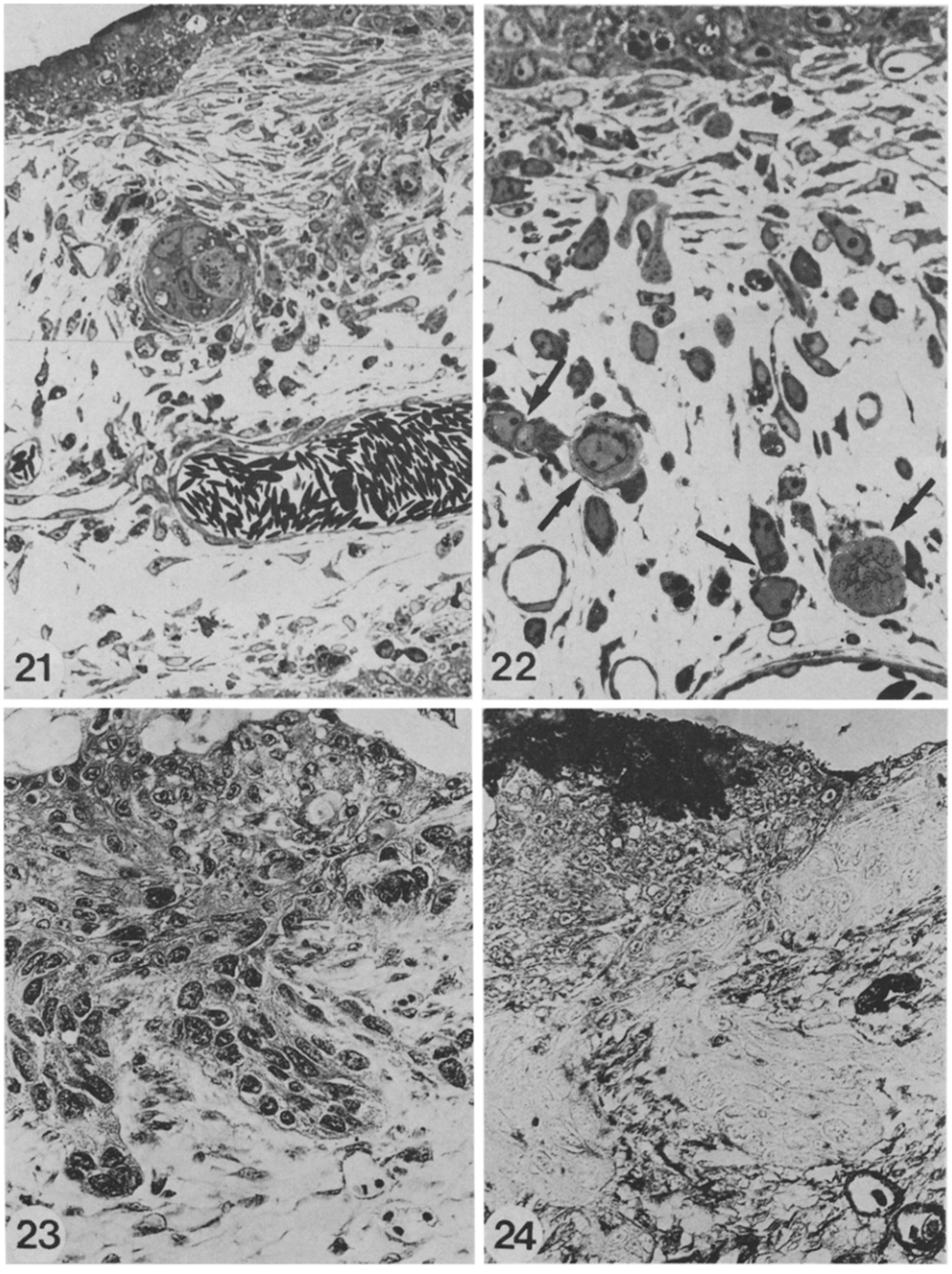


CONFRONTATION WITH EMBRYONIC CHICK HEART AGGREGATES (Figs. 17, 18 supplied by Mareel) *Cells from HN-4 (Fig. 17) and HN-1 (Fig. 18) have both penetrated into the tissue: HN-4 in solid formation, HN-1 as single cells (arrows) Histology sections* × 250 (*Fig.* 17), × 370 (*Fig.* 18).





CONFRONTATION WITH HUMAN AMNION (Figs. 19, 20 supplied by Felix) *Cells from HN-2 lie as a solid nodule on the basement membrane (Fig. 19). Several tongues from HLC-1 have invaded the mesenchyme (arrows) and have destroyed the tissue (Fig. 20).*



CONFRONTATION WITH THE CAM (Figs. 21–24 supplied by Haemmerli + Stehrenberger) *Cells from HN-3 (Fig. 21), HN-1 (Fig. 22) and HN-6 (Figs. 23, 24) have penetrated into the mesoderm. The mode of penetration is different: single cells from HN-1 indicated by arrows (Fig. 22), cell groups of HN-3 (Fig. 21), and solid tongues in the case of HN-6 (Figs. 23, 24). Application of antibody to chick tissue clearly reveals the unstained tumor cells (Fig. 24). Semithin sections: × 300 (Fig. 21), × 500 (Fig. 22). Histology sections: × 300 (Figs. 23, 24).*

detected deep within heart muscle after 4 days (Fig. 18). Solitary HN-1 cells were frequently seen within heart tissue at all stages of culture, and their destructive effects were such that some aggregates contained no identifiable cardiac elements at all after 4 weeks. HN-3, 4, 5 and 6 cells invaded the heart more slowly. The destruction of chick cardiac cells was also slower and less extensive than with HN-1 cells, and solitary tumour cells were rarely seen (Fig. 17). HN-2 cells differed markedly from all the other cell lines in that little if any infiltration into the chick heart took place.

Another model for invasion, first used by Liotta *et al.* [44], involves the use of *cultured human amnion.* The membrane consists of a single layer of epithelium attached to a continuous basement membrane with underlying dense collagenous stroma, and it provides an excellent target for studying the penetration and degradation of basement membrane by invading tumour cells.

The behaviour of HN-2 and HN-3 cells and cells from lines HTC-2 and HLC-1 and 2 on human amnion preparations were studied, using scanning and transmission electron microscopy. Some of the results have been published previously [45]. All five lines formed aggregates firmly attached to the epithelium, and subsequently established contact with the basement membrane. Three lines(HN-3, HTC-2 and HLC-1) were able to degrade the basement membrane locally. Only one line, HLC-1, displayed straightforward invasion by extending projections through the level of the basement membrane into the subjacent connective tissues. The restricted and focal pattern of basement membrane degradation, described in these *in vitro* studies, correlates closely with recent immunohistochemical investigations of Type IV collagen in surgically resected squamous carcinomas [46, 31]. Both primary invasive tumours and nodal metastases were shown to be almost completely surrounded by an intact layer of Type IV collagen, in which only focal defects were demonstrated. The behaviour of HN-2 and HLC-1 is shown in Figs. 19 and 20.

BEHAVIOUR *IN VIVO*

Chorioallantoic membrane of the chick embryo (CAM)

Cells from lines HN-1 to 6 and HTC-2 were inoculated *in ovo* onto the chorioallantoic membrane (CAM) of 10-day fertilized hens' eggs (Easty and Easty, unpublished data). The eggs were incubated for 5–7 days before removing and fixing the CAM for histological examination (Figs. 21–24). By this time, all the cell lines had penetrated between and locally dislodged the ectodermal cells of the CAM; all of them, to varying extents, had penetrated the mesoderm after 7 days. HN-1 and HTC-2 cells infiltrated the mesoderm as single cells while the other lines, particularly HN-2, were present within the mesoderm as more or less compact aggregates. HN-6 cells were frequently seen lined up as a continuous layer at the base of the ectoderm, with points of invasion from this layer on into the mesoderm. Cells from HN-5 formed highly keratinized differentiated structures within the mesoderm, closely resembling those seen in xenografts of the tumour cells in nude or immunosuppressed mice (see below). Inoculation of the CAM with suspensions of cells derived from primary cultures of normal human buccal epithelium resulted in regions of the chick ectoderm becoming intimately mixed with human epithelial cells, but the latter were not detectable within the mesoderm. Stehrenberger (unpublished data) repeated some of these experiments and, in addition, examined semithin sections of Epon-embedded material stained with an anti-chick antibody (gift from M. Marcel) which provided a clear distinction between chick and human tissues. She also studied CAMs at earlier stages. The results of Easty and Easty were confirmed: HN-1 cells infiltrated the mesoderm as single cells and formed very small aggregates on days 1 and 2, while single-cell infiltration of HN-2 did not take place. HN-6 cells grew mainly within the ectodermal layer, with occasional projections and aggregates appearing within the mesoderm after 5–6 days. Both Easty and Stehrenberger independently observed differences between the cell line HN-6 derived from a primary tongue carcinoma, and the line established from a lymph node metastasis of the same tumour. Stehrenberger found that groups of cells from the lymph node metastasis line penetrated into the mesoderm earlier than cells of the primary tumour line, while Easty observed that cells from the metastatic line tended to infiltrate under the ectodermal layer more readily than those of the primary line.

Transplantation into nude mice

Seven of the 10 lines produced solid tumours after transplantation into nude and immunosuppressed mice. In each case the histological appearance of the xenografted tumour closely resembled that of the tumour from which the line was derived. Using a monoclonal antibody specific for human type IV collagen, Gusterson *et al.* [46] reported that three of the successfully transplanted cell lines (HN-2, 5 and 6) synthesized basement membranes at the epithelial-stromal interface, with some focal discontinuities similar to those observed in surgical specimens of infiltrating squamous cell carcinomas — see above.

COMMENTS

In this review we have recorded various characteristics of cells from 10 permanent lines established from human squamous carcinomas of the

tongue and larynx. Some of the features described are common to all the lines while others are characteristic of only a few of them. In general, the common attributes reflect the derivation of the parent tumours from stratified squamous epithelium. The lines have maintained a number of epithelium-associated properties such as formation of desmosomes and tonofilaments, synthesis of cytokeratins, and production of the general epithelial markers EMA, CEA and EGF. Not every line exhibits all these features but, in spite of large differences in the expression of epithelial properties, each line possesses at least a few of them. Individual properties of the lines can be distinguished against the background of common epithelial characteristics — for example, surface configuration, ultrastructural features and social behaviour on plane substrata — which vary from one cell line to the next. To a certain extent these individual properties fall into patterns which reflect different degrees of malignancy.

Some of our studies on invasive mechanisms, notably in relation to cell motility and the release of proteolytic enzymes, illustrate this point. Cells from all the lines were motile on glass. Although there were considerable differences in the numbers of cells involved in this activity, a correlation was established between translocation on glass and invasive behaviour in experimental models. This association was most clearly exemplified by HN-1 cells. Small numbers of cells from this line migrated on glass and formed loose aggregates, and they infiltrated various 3-dimensional matrices in a characteristically dissociated single cell formation. The dispersed and infiltrative growth pattern of HN-1 cells observed in these experimental systems was closely similar to that shown by recurrent tumour in the patient, seen in tissues taken at autopsy. It is, however, interesting that the tissue in the earlier surgical resection (from which the line was derived), showed a more compact and cohesive growth pattern: the interval between the resection and the autopsy was 7 months.

Our data on proteinases are more difficult to interpret. The cell lines produced all the enzymes commonly associated with tissue degradation during invasion — cathepsin B, collagenases, elastase, plasminogen activators — but the different combination of enzyme activity found in each of the lines precluded any direct correlation with invasive patterns. Attempts to link the levels of a given protease with the behaviour of the cells in other investigations have also been unsuccessful. Destruction of biologically more realistic three-dimensional matrices is likely to prove more informative. Biochemical studies of the degradation of organized extracellular matrix by the cell lines are in progress (Burman and Carter) and, in this review, we have recorded in detail the morphological events when cells invade complex matrices such as spherical aggregates of embryonic chick heart cells, human amnion and the chick chorioallantoic membrane.

Two of the examples of local invasive mechanisms which have been presented illustrate a considerable degree of synergistic action between tumour cells and normal host elements — the interaction between tumour cells and fibroblastoid cells to enhance the formation of Type I collagenase, and the interaction of tumour cells and osteoclasts to resorb bone. In both instances, much of the destructive process is likely to be mediated by host cells, albeit primed or altered by tumour cells. Both examples may be regarded as tumour-mediated distortions of normal homeostatic mechanisms — the purely physiological process of bone remodelling by osteoclasts and the balanced process of collagen formation and collagen breakdown by fibroblasts.

In the last two models, embryonic chick heart and human amnion, carcinoma cells are confronted with organoid tissue complexes composed of epithelium, basement membrane and mesenchymal elements. Each line displayed an individual pattern of behaviour in these three systems, and it was possible to rank them according to their invasive capacity. Efficient invaders were characterized by rapid penetration into the chick heart spheroids with destruction of cardiac cells, and replacement of epithelia and spread across basement membranes in the two organoid models. One of the cell lines (HN-2) failed to penetrate into the spheroids and traverse basement membranes.

One striking feature that has emerged from these various investigations is the stability of both the common and individual properties of the cell lines. During observation periods of up to 6 years a number of features such as ultrastructural morphology, motility and infiltration of CAMs were repeatedly examined and found to be unchanged. No trend towards morphological differentiation and/or behavioural convergence have become apparent, and the cell lines continue to display a degree of biological diversity which, given the phenotypic stability of the lines, is unlikely to be due to prolonged growth *in vitro*.

A final comment may be made on the scope for correlation of *in vitro* properties with *in vivo* clinical and pathological features. The strong selective pressures involved in the establishment and maintenance of tumour cell lines of any kind do not need to be emphasized, nor the functional isolation of such lines, completely separated from any form of modification by host elements. On the other

hand, each of the lines studied here has shown considerable phenotypic stability. Broad correlations can be adduced for several of the lines between their growth patterns in the original tumours and their mode of growth in various experimental systems. The highly aggressive HN-1 cell line may be cited as an example. The patient from whom it was derived developed recurrent tumour shortly before he died which showed a diffuse single cell growth pattern. The single cell state is a prerequisite for locomotion which in turn is an index of enhanced invasive capacity; and it is striking that HN-1 displayed the same dissociated mode of invasion in various three-dimensional experimental matrices. Since the line was established from an earlier surgical resection in which the tumour showed a compact, aggregated growth pattern, it is possible to interpret the change from a compact to a dissociated arrangement as evidence of tumour progression, an event which (in this instance) was demonstrated both in tumour growing in the patient and in culture. Interesting questions are raised as to the possible applications of *in vitro* assays to demonstrate or even predict at least some aspects of invasive behaviour. Exposure to experimental conditions may, however, accelerate or amplify the inherent capacities of tumour cell populations, and we are confronted at this point with the problem of actual or manifest tumour invasion and invasive potential [47] — a difference which is likely to be determined to a large extent by the presence or absence of a reacting host organism.

REFERENCES

1. Easty DM, Easty GC, Carter RL, Monaghan P, Butler LJ. Ten human carcinoma cell lines derived from squamous carcinomas of the head and neck. *Br J Cancer* 1981, **43**, 772–785.
2. Haemmerli G, Sträuli P. *In vitro* motility of cells from human epidermoid carcinomas. A study by phase-contrast and reflection-contrast cinematography. *Int J Cancer* 1981, **27**, 603–610.
3. Haemmerli G, Arnold B, Sträuli P. Cellular motility on glass and in tissues: Similarities and dissimilarities. *Cell Biol Int Rep* 1983, **7**, 709–725.
4. Haemmerli G, Jockusch BM, Sträuli P. Cellular motility and cytoskeletal arrangement in human squamous carcinoma cells. *J Submicrosc Cytol* 1984, **16**, 169–170.
5. Monaghan P, Knight J, Cowley G, Gusterson BA. Differentiation of a squamous carcinoma cell line in culture and tumorigenicity in immunologically incompetent mice. *Virchows Arch Cell Pathol* 1983, **400**, 87–95.
6. Knight J, Gusterson BA, Cowley G, Monaghan P. Differentiation of normal and malignant squamous epithelium *in vivo* and *in vitro* — a morphological study. *Ultrastruct Pathol* 1984, **7**, 133–143.
7. Haemmerli G, Felix H. Interrelation of motility, shape, and surface architecture in human carcinoma cells. *Cell Biol Int Rep* 1981, **5**, 929–936.
8. Haemmerli G, Felix H. Shape and motility, two interdependent features. Scanning Electron Microscopy 1982, **2**, 731–739.
9. Hauser-Urfer IH, Stauffer J. Comparative chromosome analysis of nine squamous cell carcinoma lines from tumors of the head and neck. *Cytogen Cell Genet* 1985, **39**, 35–39.
10. Knight JC. An experimental model for human keratinocyte differentiation: Generation and characterisation of monoclonal antibody markers. (Thesis) University of London. 1984.
11. Knight J, Gusterson B, Russel Jones R, Wilson P. Monoclonal antibodies specific for subsets of epidermal keratins: Biochemical and immuno-cytochemical characterisation — application in pathology and cell culture. *J Pathol* 1985, **145**, 341–354.
12. Sloane JP, Ormerod MG. Distribution of epithelial membrane antigen in normal and neoplastic tissues and its value in diagnostic tumor pathology. *Cancer* 1981, **47**, 1786–1795.
13. Ormerod MG, Monaghan P, Easty DM, Easty GC. Asymmetrical distribution of epithelial membrane antigen on the plasma membranes of human breast cell lines in culture. *Diagn Histopath* 1981, **4**, 129–●.
14. Ellison ML. Tissue culture. In: Symington T, Williams AE, McVie JG, eds. *Cancer Assessment and Monitoring*. Edinburgh, London, New York, Churchill-Livingstone, 1980, 59–66.
15. Cowley G, Smith JA, Ellison M, Gusterson B. Production of beta-human chorionic gonadotrophin by human squamous carcinoma cell lines. *Int J Cancer* 1985, **35**, 575–579.
16. Cowley G, Gusterson BA, Smith J, Hendler F, Ozanne B. The amount of EGF receptor is elevated on squamous cell carcinomas. *Cancer Cell* 1. In: *Cold Spring Harbor Meeting. The Transformed Phenotype*. 1984, 5–10.
17. Cowley GP, Smith JA, Gusterson BA. Increased EGF receptors on human squamous carcinoma cell lines. *Br J Cancer* (in press).
18. Ozanne B, Richards CS, Cassels D, Shum A, Grossman D, Trent J, Gusterson B, Hendler F. Evidence for increased EGF receptor in epidermoid malignancies. In: *Cold Spring Harbor Meeting. Cell Proliferation Symposium* (in press).

19. Pfyffer GE, Haemmerli G, Heizmann CW. Calcium-binding proteins in human carcinoma cell-lines. *Proc Natl Acad Sci USA* 1984, **81**, 6632–6636.
20. Heizmann CW, Berchtold MW, Rowlerson AM. Correlation of parvalbumin concentration with relaxation speed in mammalian muscles. *Proc Natl Acad Sci USA* 1982, **79**, 7243–7247.
21. Stuhlfarth J, Reininghaus J, Jockusch H, Heizmann CW. Calcium-binding protein, parvalbumin, is reduced in mutant mammalian muscle with abnormal contractile properties. *Proc Natl Acad Sci USA* 1984, **81**, 4814–4818.
22. MacManus JP, Whitefield JF, Boynton AL, Durkin JP, Swieringa SHH. Oncomodulin — a widely distributed tumor specific, calcium-binding protein. *Oncodev Biol Med* 1983, **30**, 79–90.
23. Haglid KG, Stavrou D, Ronnback L, Carlsson CA, Weidenback W. The S-100 protein in water-soluble and pentanol-extractable form in normal human brain and tumours of the human nervous system. *J Neurol Sci* 1983, **20**, 103–111.
24. Gaynor R, Irie R, Morton D, Herschmann HR. S-100 protein is present in cultured human malignant melanomas. *Nature (London)* 1980, **286**, 400–401.
25. Baici A, Sträuli P. Release of proteinases by cultures of human cell lines derived from squamous carcinomas of the tongue and larynx. *Exp Cell Biol* 1985, **53**, 213–219.
26. Burman JF, Carter RL. Lysis of Type I collagen by squamous carcinomas of the head and neck. *Int J Cancer* (in press).
27. Tsao S-W, Burman JF, Easty DM, Easty GC, Carter RL. Some mechanisms of local bone destruction by squamous carcinomas of the head and neck. *Br J Cancer* 1981, **43**, 392–401.
28. Tsao S-W, Burman JF, Carter RL. Hypercalcaemia and *in vitro* osteolysis associated with xenografts of squamous carcinomas of the tongue. *Br J Cancer* 1983, **48**, 103–107.
29. Tsao S-W, Burman JF, Pittam MR, Carter RL. Further observations of mechanisms of bone destruction by squamous carcinomas of the head and neck: The role of host stroma. *Br J Cancer* 1983, **48**, 697–704.
30. Carter RL, Tsao S-W, Burman JF, Pittam MR, Clifford P, Shaw HJ. Patterns and mechanisms of bone invasion by squamous carcinomas of the head and neck. *Am J Surg* 1983, **146**, 451–455.
31. Carter RL, Burman JF, Barr L, Gusterson BA. Immunohistochemical localization of basement membrane Type IV collagen in invasive and metastatic squamous carcinomas of the head and neck. *J Pathol* (in press).
32. Dontenwill W, Chevalier HJ, Reckzeh G. Growth of carcinomas in the region of the cartilage. *J Natl Cancer Inst* 1973, **50**, 291–295.
33. De Ridder L, Mareel MM, Vakaet L. Resistance du cartilage embryonnaire à l'invasion par des cellules tumorales *in vitro*. *C R Soc Biol* 1973, **167**, 1688–1689.
34. Easty DM, Easty GC. Measurement of the ability of cells to infiltrate normal tissues *in vitro*. *Br J Cancer* 1974, **29**, 36–49.
35. Kuettner KE, Pauli BU, Sable L. Morphological studies on the resistance of cartilage to invasion by osteosarcoma cells *in vitro* and *in vivo*. *Cancer Res* 1978, **38**, 277–287.
36. Carter RL, Tanner NSB. Local invasion by laryngeal carcinoma: The importance of focal (metaplastic) ossification within laryngeal cartilage. *Clin Otolaryngol* 1979, **4**, 283–290.
37. Pittam MR, Carter RL. Framework invasion by laryngeal carcinoma. *Head Neck Surg* 1982, **4**, 200–208.
38. Carter RL. Patterns and mechanisms of localized bone invasion by tumors: Studies with squamous carcinomas of the head and neck. *CRC Critical Reviews in Clinical Laboratory Sciences* (in press).
39. Pauli BU, Memoli VA, Kuettner KE. Regulation of tumor invasion by cartilage-derived anti-invasive factor *in vitro*. *J Natl Cancer Inst* 1981, **67**, 65–74.
40. Bracke ME, Van Cauwenberge RM-L, Mareel MM. Interaction of malignant cells with salt-extracted cartilage *in vitro*. *Cancer Res* 1984, **44**, 297–304.
41. Easty GC, Haemmerli G, Easty DM, Sträuli P. Interactions between normal epithelial and squamous carcinoma cells in monolayer culture. *Cancer Res* 1982, **42**, 4248–4255.
42. Mareel MM, Kint J, Meyvisch C. Methods of study of the invasion of malignant C^3H-mouse fibroblasts into embryonic chick heart *in vitro*. *Virchow's Arch B Cell Path* 1979, **30**, 95–111.
43. Mareel MM, De Bruyne GK, Vandesande F, Dragonetti C. Immunohistochemical study of embryonic chick heart invaded by malignant cells in three-dimensional culture. *Invas Metastas* 1981, **1**, 195–204.
44. Liotta LA, Lee CW, Morakis DJ. New method for preparing large surfaces of intact human basement membrane for tumor invasion studies. *Cancer Lett.* 1980, **11**, 141–152.
45. Felix H, Tchao R, Schleich A, Hoffmann V. Interactions of tumor cells with human amnion membrane: a model for studying tumor invasion *in vitro*. *Scanning Electron Micros* 1982, **2**, 741–749.
46. Gusterson BA, Warburton MJ, Mitchell D, Kraft N, Hancock WW. Invading squamous cell carcinoma can retain a basal lamina. An immunohistochemical study using a monoclonal antibody to Type IV collagen. *Lab Invest* 1984, **51**, 82–87.
47. Sträuli P. Invasive potential and actual invasion: An essay on tumor progression. Jahrbuch der Schweiz. *Naturforsch Gesellschaft* 1985.