PRIMARY TISSUE CULTURE OF HUMAN LARYNGEAL CARCINOMA AND INTERACTION WITH NATIVE LYMPHOCYTES

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ABSTRACT

Primary tissue culture of human laryngeal carcinomas, producing cells similar to the original tumour, was effected in serum-supplemented medium. This culture method provided a good investigative model for the study of lymphocyte/epithelial cell interactions in tumour and normal cultures.

Of 107 laryngeal and hypopharyngeal carcinomas, 45% grew. The appearances were compared with similar preparations taken from normal true cord of the same larynx, of which 70% grew. No morphological differences were found by light or electron microscopy. Bizarre morphology occurred in 20% of cultures, mainly in tumour but also in normal cultures. This appeared in cultures which had ceased proliferation at an earlier stage than non-bizarre cultures and was identified by inactivity of monolayer and lack of intracellular activity, as observed by time-lapse video microscopy.

Cultures were revealed by time-lapse video microscopy to support motile lymphocytes, which appeared phase-dark, both on the upper and lower monolayer surfaces under phase-contrast optics. Tumour-infiltrating lymphocytes migrated from laryngeal explants onto the emerging monolayer. These cells remained activated on the culture for as long as 19 days. Many attempts were made by tracking methods to identify lymphocyte phenotype, which was found to be T cell, although heterogeneous subset identities existed. Bizarre type cultures lacked motile lymphoid cells suggesting that lymphocytes require stimulants produced by proliferating cultured epithelial cells. Mitotic tumour cells were shown, using vector analysis, to be chemotactic for T cells. This was a unique function of the culture method. This chemotactic phenomenon could not be repeated if diffusion was increased, presumably because products of cell growth were dissipated.

Mitotic chemotaxis may be related to mediators produced by the epithelium specific for T cells.

THIS THESIS IS DEDICATED TO MY FAMILY AND FRIENDS

AND TO THE MEMORY OF DR PETER SOARES

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QUOTATION

'Motility in cells must imply a special function ; we are in no doubt what that function is in the case of spermatozoa, polymorphs and monocytes. When motility ceases we must assume that the objective has been attained, as journey's end in lovers' meeting. The cliché 'round cell infiltration' establishes the fact that the wanderings of the lymphocyte are not invariably aimless, but that under certain conditions they clearly reach their destination and cease to move.

.....they move towards certain cells, and remain attached for long periods; if they leave they return again; their cytoplasm licks, as it were, the cytoplasm of other cells; in the cases shown these are malignant cells. No other motile cell does this; for example the polymorph, the eosinophil and the monocyte never attach themselves to other cells in this way. It is true that we cannot deviate the lymphocyte from its apparently purposeless wandering by any chemical bait or by small particulate objects as we can so readily do in the case of the monocyte and polymorph.

.....When you see the lymphocyte moving in a time-lapse film you may be reminded of Graves' poem on the cabbage white butterfly:

'The butterfly, the cabbage white,
(His honest idiocy of flight)
Will never now, it is too late
Master the art of flying straight.'

On the other hand, on occasions when the lymphocyte approaches close to certain other cells its movements are directed towards them. Dare we introduce a new word for this and call it 'cytotropism'?'.

Professor R J V Pulvertaft, O B E (1959).

CHAPTER 1

INTRODUCTION

INTRODUCTION

With inadequate culture methods due to lack of understanding of the optimum growth medium, it is not surprising that difficulty has frequently been experienced in growing squamous carcinomas *in vitro*. Carcinomas of the head and neck are especially difficult to grow compared with other tumours. As the field of tissue culture has developed and it was realised that epithelial cells and their tumours have special growth requirements, fresh attempts at culture of carcinomas were made. These are described in Chapter 2, which is a review of all the relevant literature including tissue culture. The primary aim of this project was to culture squamous cell carcinoma of the human larynx using improved methods.

Laryngeal cancer comprises a variety of different types of neoplasm, but 97% of cases are squamous cell carcinoma. The most common malignant tumour in the head and neck region and also the whole body is squamous cell carcinoma. Squamous cell carcinoma of the larynx and hypopharynx arises from the thin lining of epithelial mucosa covering intralaryngeal and extralaryngeal structures, respectively. In the United Kingdom and many other countries, the vocal cords are most commonly affected. For this reason control epithelium for culture was taken from non-neoplastic true cord. Early stage tumours were not included in this study, since laryngectomy is seldom required for treatment, irradiation treatment usually being successful to cure the lesion. Total laryngectomy or gastric pull-up operations were carried out on advanced tumours, and after tumours which had been irradiated, had failed to respond. The neoplastic material available for culture was thus most often an advanced tumour. This

required extra adaptations to be made to the culture technique, which will be described.

In Chapter 3, cultures of carcinoma were compared with normal control cultures, grown under identical experimental conditions. Results were assessed for the influence of pre-operative irradiation therapy on proliferation of cells. Characteristics of cytokeratin formation and cell morphology and surface structure were investigated by immunocytochemistry and electron microscopy, respectively.

An advantage of the explant method is the heterogeneity of cell types which can be cultured. In particular time-lapse video microscopy showed that lymphocytes migrated out of explants and interacted with the proliferating culture. In Chapter 4 lymphocyte motility is described and observations made about 1) the apparent need for a viable co-culture substratum for motility to occur and 2) a 'sandwich culture' method to prevent dissipation of the stimuli for chemotaxis of lymphocytes to mitotic carcinoma and normal cells to occur.

Chapter 5 investigates the plane of movement of the lymphocytes. Do they move on the apical surface of the tumour culture or rather basolaterally so that they underlap the culture? Tests were carried out to assess whether lymphocytes were clinging to the apical surface of cultures. This involved the application of a matrix into which lymphocytes would quickly and preferentially migrate. Further evidence of apical movement and also emperipolesis was obtained by time-lapse video microscopy.

Chapter 6 attempts to identify the subset phenotype of motile lymphocytes on cultures. Infiltrating lymphocytes in sectioned tissue and

lymphocytes in explant tissue vasculature have been stained to identify some but not all to phenotype level.

In Chapter 7, an investigation by a statistical method called vector analysis is described to test for chemotaxis of lymphocytes to mitotic cells in cultures of carcinoma and normal laryngeal epithelium.

Chapter 8 is a conclusion of all findings in this thesis. This is followed by a Bibliography of cited references and Appendices 1, 2 & 3.

A Published Paper, Wang *et al*, (1986) pertaining to some of the work presented in this thesis is included in the inside back cover of the thesis.

A VHS Video Presentation is contained in this thesis. It is a short demonstration lasting approximately 15 minutes, of lymphocyte locomotion. The video may be played independently of this text at any appropriate moment on a standard VHS video cassette recorder. A simultaneous narration is recorded on the video. For the purposes of playing back and reviewing video sequences, a time-code is displayed on the monitor screen during play. This is situated in the centre of the lower edge of the screen.

CHAPTER 2

LITERATURE REVIEW

LITERATURE REVIEW

The purpose of this literature review is to present an introduction to the theme of this thesis. It will describe attempts by other workers to grow carcinomas *in vitro* and show that at the commencement of this study there was a need for a suitable culture system for human laryngeal squamous carcinoma. Further sections proceed to review the literature relevant to the applications of this growth model, which are described in Chapters 3, 4, 5, 6 and 7. The review also covers several topics relating to lymphocytes. The identities of lymphocytes within normal and diseased skin and carcinomas are reviewed, in an attempt to identify lymphocytes motile on cultures. Studies of lymphocyte locomotion while in contact with other non-lymphoid cells and in chemotaxis assays are described. Motile responses of lymphocytes as a characteristic of phenotype and function and as an immunological reaction are discussed. In particular a broad assessment is presented of humoral and cellular stimuli likely to be present during the culture of laryngeal epithelial and carcinoma cells by explant method.

TISSUE CULTURE BY EXPLANT METHOD

This study of laryngeal carcinoma in tissue culture by the explant method is a novel one. The explant method requires the transplantation of a tissue fragment from the living body to a laboratory environment designed for the purpose of culture. The excised tissue becomes an 'explant', which is secured in place either in a fibrin clot or simply in a drop of medium held between plastic or glass supports. Essential growth

medium bathes the explant, while the temperature and gaseous content of the environment are controlled at its physiological level. Explants receive no further manipulation or any enzymatic treatment. In the explant method the tissue architecture is thus retained intact, unlike the method of cell culture which dissociates cells into aggregates and single cells. This is a fundamental difference which is important to the topic of this thesis. Explants produce cultures comprising cell types as heterogeneous as the original tissue explant. Consequently, explant tissue culture should be more representative of the original tissue than other types of culture.

A Brief Historical Overview

Harrison is considered to be the founder of tissue culture. In 1907, he was the first to apply the explant method as a tool (Harrison, 1907). He demonstrated that nerve fibres develop from frog neuronal perikarya. The method he chose was the hanging drop technique. Tissue was embedded in a lymph clot on a glass slide, which was inverted over a small indentation in a second slide. A drawback of this method was that growth medium could not be changed so that culture time was short. Nevertheless, explanted epithelium from the guinea pig was first grown by hanging drop technique in 1917 by Loeb and Fleisher.

Flask Culture

Flask culture was introduced by Carrel in 1923. His flask had a short neck angled to facilitate replacement of nutrients and is still widely used today. Replacing exhausted medium with fresh medium enabled cultures to be grown for longer periods than by the hanging drop method.

Explants were made to adhere to the flask surface by embedding in plasma clots. This not only secured the explant in place but provided protection when renewing medium. Carrel described a number of explant cultured tissues, but omitted to mention the origins of the tissues. However, his study has particular relevance to the topic of this thesis, since it included the co-culture of epithelial cells, fibroblasts and leucocytes. Carrel's work demonstrated the versatility of the explant method for various tissues, particularly epithelium. Furthermore, his results served to promote the benefits of this type of system to the author during the initial project design for this study. The scope of his findings were broad; ranging from the observation that fibroblasts proliferated faster than epithelial cells to the finding that resting fibroblasts were stimulated to proliferate by contact with 'wandering' spleen cells (presumably lymphocytes).

More recently the explant method has been helpful in studying migration and differentiation of epithelial cells during wound repair by Resau *et al*, (1984). This indicated that it was an excellent method for demonstrating epithelial cell migration, relating well to the physiological repair mechanism. As migration is a characteristic of cancer cells, it seemed that primary explant culture might therefore be a good tool for the investigation of carcinomas.

Explant Culture of Carcinomas

In my survey of the literature I was specifically interested in studies of carcinoma explant culture. At the commencement of this work there existed only a few reports of this nature. Krause *et al*, (1981) and Easty *et al*, (1981) reported a surface culture method in which explants

were grown on the flask surface. They found that fibroblast outgrowth was detrimental to epithelial colonies. No study was carried out on the explant cultures *per se*. The cultures (some from laryngeal carcinoma) were passaged to form cell lines. Edwards *et al*, (1980) comment on the problems encountered when growing explants of head and neck cancer. They reported a mixture of cells growing out from carcinoma explants comprising fibroblasts, normal epithelial cells and carcinomatous cells. They used a monoclonal antibody to kill contaminating fibroblasts of several tumour explants, including two laryngeal carcinomas. More recently, Freeman *et al*, (1986) have cultured primary explants of squamous cell carcinoma of the breast, lung and stomach on collagen gels. At the present time of writing, the study by the author was the only other report of the explant method for actually studying proliferating carcinomas (Wang *et al*, 1986). A copy of this reference is bound in the inside back cover of this thesis.

In search of a broader related bibliography, a literature search was focused on explant culture of normal epithelium, since carcinoma arises by changes in such epithelium. The literature provided several examples of the explant method for skin research. This method has proven useful for a variety of purposes such as investigating the effect of allergens (Karasek, 1966), the processes of wound healing (Vocci *et al*, 1983), the effect of vitamins and cortisol (Prose *et al*, 1967), the proliferation of psoriasis (Pullman *et al*, 1974) and the effect of carcinogens (Steel *et al*, 1978). Explant cultures performed well in carcinogenesis studies as a result of the presence of dermis, which exerted powerful influences on epidermal carcinogenesis (Pinto *et al*, 1968).

The sparsity of reports in the literature of carcinoma culture grown by explant method is attributable to a combination of three factors:

1) the inhibitory effect of fibroblasts on carcinoma cells in culture;

2) primary carcinoma cultures have been grown by an alternative culture method most accurately described as cell culture. Their subsequent subcultivation has produced some well established cell lines. The best known is HeLa from a carcinoma of the cervix. Two laryngeal carcinomas produced the laryngeal cell line HEp-2 (Moore *et al*, 1955). Foley *et al*, (1960) developed CCRF-108, another laryngeal cell line. Easty *et al* (1981) established HN-2, HN-4, HN-8 and HN-10 cell lines from squamous carcinoma of the larynx.

3) The employment of the explant method for primary culture has hitherto had a low success rate for reasons which will be described.

The awareness of these difficulties indicated that the majority of carcinomas could not be grown by the explant method.

Feeder Cell Layer

A novel approach to explant culture of carcinomas came as the result of a major development in the culture of normal epithelium. This was the introduction of a feeder layer comprising murine fibroblasts (Rheinwald & Green, 1975). It was used to enhance epithelial cell growth before fibroblasts from the connective tissue proliferated. Prior to 1975, the explant culture of epithelium was hampered by overgrowth of fibroblasts. Efforts were made to inhibit fibroblast growth to allow the slower growing epithelial cells to grow, but were only moderately successful (Dover, 1985). An alternative approach was to enhance the growth of epithelial cells. Rheinwald and Green (1975) showed that epithelial cell growth was enriched by the presence of an irradiated feeder layer of 3T3 fibroblasts.

Fibroblasts were stopped from proliferating by irradiation, but stayed alive and continued to secrete growth factors into the medium. This emphasised the special needs growing epithelium has for growth factors. The same method was successful for growing explant cultures of squamous carcinomas for subsequent cell line establishment (Rheinwald and Beckett, 1981), but no studies were carried out using the explant method.

As the explant method without the complexities of a feeder layer was unsatisfactory for the culture of human carcinomas, it was necessary to seek help from the literature regarding alternative methods of primary culture of squamous carcinomas grown using cell culture techniques. Cobleigh *et al*, (1987) grew squamous cell carcinoma on feeder layers but chose to study only dissociated cells from carcinomas of the head and neck, rather than explants. These authors concluded that 3T3 fibroblast support increases the number of successfully grown carcinoma cultures by approximately two-fold. They also found that higher plating densities of carcinoma cells produced more successful cultures when grown with or without a feeder layer than did low plating densities. It seemed that explants may produce the highest plating density of all, even to the extent of growing well without a feeder layer support. This was found, indeed to be the case for laryngeal carcinoma explants (Wang *et al*, 1986). This will be described in this thesis.

Growth Factors

Enhancement of epithelial cell growth in the absence of a feeder layer followed after the contribution of the feeder layer was understood. It was found that the inclusion of epidermal growth factor (Rheinwald & Green, 1977) and cholera toxin (Green, 1978) had the same effect as a

feeder layer. This alternative was adopted for the early work on culture of laryngeal carcinomas reported in this thesis. Growth and metabolism of most mammalian cells in culture require that growth factors, hormones and other factors be present in the basal medium. Typically, these factors are provided exogenously by supplementing basal medium with fetal bovine serum and/or other sera usually used in cell culture protocols. A number of growth factors and hormones have been identified, isolated and purified from a variety of sources, including animal tissues and cellular secreted products; others are produced synthetically. Growth factors and hormones available from suppliers of reagents can be used in conjunction with other essential nutrients to reduce or eliminate the need for commercially prepared so-called 'serum replacements'.

Serum Selection

Laryngeal carcinoma cultures required undefined growth factors present in selected batches of sera which were added to growth medium. The ability of serum to support growth of carcinoma culture is variable from batch to batch. The importance of pre-selecting serum for optimal growth promotion of normal keratinocytes was mentioned by Rheinwald and Beckett, (1981). This regimen was used by the author for growing laryngeal cultures.

Anchorage Dependence

Human head and neck carcinomas have been grown by a variety of cell culture methods. Some earlier methods are not now valid. Mattox and Von Hoff, (1980) used soft agar culture. At the time, this method was considered acceptable because colony formation in agar was one of the

accepted criteria of tumorigenicity, distinguishing transformed tumour cells from normal cells. Furthermore, the method was selective against fibroblasts which are anchorage dependent and unable to form colonies in agar. However, Rupniak and Hill (1980) pointed out the unsuitability of this method for head and neck squamous carcinomas. Rheinwald and Beckett (1981) also found poor colony formation on semi-solid methylcellulose support. Furthermore certain squamous cell carcinoma cell lines resemble normal keratinocytes in the characteristic that they are triggered to differentiate terminally if deprived of anchorage (Green, 1978 and Rheinwald *et al*, 1977). Dodson *et al*, (1981) explained that carcinomas and squamous carcinomas in particular, capable of growing monolayer cultures, retained a degree of anchorage dependence. Cobleigh *et al*, in 1987, confirmed that head and neck carcinomas were strictly anchorage dependent. This problem could be overcome by a variety of anchorage methods. Embedding the explant in plasma clots was a popular method as already mentioned. Another method was to allow the explants to dry slightly on the surface of the flask until they lost their wet appearance, prior to adding medium (Fischer *et al*, 1980).

Attachment Factors

Attachment factors enhance the attachment and spreading of many types of anchorage dependent cells. Some cell types can synthesize their own attachment factors while others cannot produce enough to adhere and grow. By supplementing attachment factors to the cell culture system, cells which do not attach well to glass or plastic show greatly improved plating efficiency.

Serum

Serum contains many components which promote cell attachment and spreading. When using serum-free media it is often necessary to replace these serum components with attachment factors (Maurer, 1986).

Extracellular Matrix

Extracellular matrix components influence attachment, cell spreading, growth, morphology, differentiation and motility of many cells. Exogenous addition of these components to cultures can be of benefit (Kleinman, 1987).

Surface Charge Interactions

Electrostatic interactions occur between the negatively charged surface ions of the cell membrane and the positively charged surface ions of attachment factors on the substratum. Poly-L-lysine, fibronectin or collagen can be absorbed to the surface to increase the number of positively charged sites available for cell binding.

Culture Difficulties of Head and Neck Carcinomas

The culture of head and neck cancer is the most difficult among all human squamous carcinomas. In addition to inhibitory effects of fibroblast and microbial contamination, a further problem occurs if the tumour is dissociated. This has been identified by cell culture studies of Cobleigh *et al*, (1986). They reported that a cell population is present in head and neck carcinomas, which inhibits colony formation during culture

of dissociated cells. This suggests that dissociated culture techniques are inappropriate for the culture of head and neck carcinomas.

From the literature review it can be seen that explant culture of carcinomas has the following requirements:

1) the possibility of extended culture

2) the control of fibroblasts

3) growth factors or a feeder layer; the presence of active serum factors determined by pre-screening

4) anchorage to a substratum.

LYMPHOCYTES WITHIN TISSUE EXPLANTS

I have observed that lymphocytes migrated from laryngeal carcinoma explants to interact with the proliferating carcinoma cultures. Similarly lymphocytes migrated out of normal control explants. In Chapter 6, methods are described to locate and identify motile lymphocytes after fixation. The recent literature was consulted to find out which groups of lymphocytes infiltrate carcinoma, normal skin and mucosal explants and thereby suggest the types of monoclonal antibodies to select for identifying motile lymphocytes. This was relevant to the procedures adopted in Chapter 6.

Lymphocytes in Blood Vessels

Capillary blood vessels in explants of carcinomas are a likely source of motile lymphocytes on cultures. Miodonski *et al*, (1980) found that the vascular structure of advanced laryngeal carcinomas differed within three zones. These are 1) the interior of the tumour, 2) the tumour border where tumour growth is most intense and 3) adjacent tissue to the tumour. The peripheral portions of the tumour (especially small tumours growing out of the true mass of an exophytic neoplasm), contained the largest collection of capillaries. Within the deeper tumour strata there were avascular regions of varying size. These corresponded to the larger foci of neoplastic cells or to places of necrotic destruction. Tumour explants may vary in vascularisation. Thus, the amount of motile lymphocytes derived from vessels within explants would depend upon the extent of

vascularisation. Presumably an explant with a dense concentration of vessels would release many lymphocytes from its cut edges.

Lymphocytic Infiltrates

Lymphocytes are present in normal tissue and in many pathological lesions. Their presence as infiltrates in dermatoses, solid tumours, normal skin and mucosae has been quantitatively analysed by Bos *et al*, (1986) and Smolle (1988).

Tumour Infiltrates

Tumour infiltration by lymphocytes is particularly common in naturally-occurring human tumours and experimentally-induced animal neoplasia. Ehrlich in 1909 observed that lymphocytes accumulate at the peritumoural interface with the host and realised that this was an immune response to the tumour. Smolle (1988) indicated that infiltrates in squamous cell carcinomas, malignant melanoma and seborrhoeic keratosis were similarly based. He described a T cell compartment which is common to all these lesions. Other immunohistological studies confirmed that the majority of mononuclear infiltrates in solid tumour were T lymphocytes (Poppema *et al*, 1983; Lennard *et al*, 1984).

The significance of lymphocyte infiltration in tumours will be assessed since in certain cancers a favourable prognosis has been linked to lymphocyte infiltration. My model of motile tumour-infiltrating lymphocytes may thus have potential application in the research of the

mode of infiltration into solid tumours. The effect of the lymphocyte infiltrate on the growth of the tumour which it infiltrates, has always been contentious. Very few cancers seem to be restricted by a moderate mononuclear cell infiltrate. The exceptions are medullary carcinoma of the breast and perhaps seminoma of the testis. Heavy infiltration of lymphocytes in tumour stroma does in the latter appear to carry a more favourable prognosis. This is sometimes also true of carcinomas of the breast, bladder, stomach and cervix and malignant melanoma, choriocarcinoma and neuroblastoma (Kreider *et al*, 1984). Laryngeal carcinoma has been assessed for prognostic influence of infiltrating leucocytes by many workers (Sala *et al*, 1976 and Bennett *et al*, 1971). Their results are not in agreement, except in the finding that there is no overwhelming correlation of good prognosis with leucocyte infiltration at all stages of the disease. Bennett *et al* found that certain stages (Grade I and II) of well-differentiated tumours did have a better survival rate. However their finding that poorly differentiated tumours with infiltration have an extended survival rate is contradicted by Sala *et al* who found that all poorly differentiated tumours have poor prognosis regardless of some infiltration or none. At that time (1977) it was impossible to identify lymphocyte subsets beyond classification into B cells or T cells. This prevented a fuller understanding of infiltrating lymphocytes and their functions.

Lymphocyte Phenotypes

The advent of monoclonal antibodies has enabled lymphocytes to be categorised according to their immunological phenotype. Lymphocyte

phenotypes in human breast carcinoma have been identified and quantified by Rowe *et al*, (1984). They found that lymphocytes had accumulated in numbers which did not reflect the simple recruitment from peripheral blood. There was a higher ratio of suppressor/cytotoxic cells to helper cells than found in peripheral blood, raising the question as to whether infiltrating lymphocytes can proliferate in the tumour or whether they are selectively recruited from peripheral blood. This study was useful in showing the correlation of higher lymphocyte numbers infiltrating carcinoma of the breast than found in normal or benign conditions. Breast tumour infiltration did not appear to be associated with activated T cells, in contrast to other conditions, such as polymyositis and muscular dystrophy (Rowe *et al*, 1983).

Infiltrates in Carcinoma of the Tongue

A profile of immunocompetent cell infiltrates, in squamous carcinoma of the tongue is reported by Boscia *et al*, (1988). The majority of infiltrating mononuclear cells were activated T lymphocytes, in contrast to the findings in breast cancer by Rowe *et al* (1984). Helper/inducer T cells predominated in the tumour stroma, but in the parenchyma, suppressor/cytotoxic T cells were more numerous. Natural killer cells were infrequent and B cells and monocytes were rare.

Lymphocytic Infiltrates in Laryngeal Carcinoma

A similar study of human laryngeal carcinomas was carried out by Zeromski *et al*, (1988). They found that laryngeal carcinoma was

infiltrated by a highly heterogeneous cell population. Small numbers of suppressor/cytotoxic T cells and natural killer cells were present within the tumour. Here the absence of helper/inducer T cells could account for failed anti-tumour action by cytotoxic cells. Helper/inducer T cells accumulated at the tumour periphery. This indicated to the author of this thesis that a range of lymphocytes may be present in laryngeal carcinomas, which may differ between different laryngeal carcinomas.

Effects of Infiltrating Lymphocytes

A co-culture model and an organ culture model showing the effect of infiltrating lymphocytes have been described suggesting that lymphocyte interaction with carcinoma *in vitro* may reflect the situation *in vivo*. Evidence of stimulatory effects of lymphocytes on tissue was found. (The stimulatory effect of motile lymphocytes on resting fibroblasts as shown by Carrel, 1923, was mentioned earlier). Gorlina *et al*, (1987) describe the differentiation of fibroblasts caused by contact with lymphocytes. Lymphocytes were found to regulate regeneration and proliferation of connective tissue. MacDonald and Spencer (1988) demonstrated that activated T cells within gut mucosal explants cause crypt epithelial cell hyperplasia and villous atrophy. The mechanism of this reaction is unknown. If T cells can induce epithelial cell division it is a possibility that there is some similar interaction in tumours.

Functional Responses of Isolated Tumour-Infiltrating Lymphocytes

Since motile lymphocytes on cultures were likely to be tumour-infiltrating lymphocytes (TIL) the literature regarding TIL was reviewed for relevant studies. Functional tests of TIL were compared to those of peripheral blood lymphocytes (PBL) which are often used in tests with tumour cells devised to resemble lymphocytes infiltrating tumours. These studies show that neither isolated TIL nor PBL provide satisfactory lymphocyte models of lymphocyte infiltrates *in situ*. Several studies on **human** tumour-infiltrating lymphocytes (TIL) have been performed, some of which will be mentioned in this section. TIL recovered from tumours and studied in bulk cultures were found to be poor effectors of cytotoxicity in *in vitro* assays with different tumour targets (Vose *et al*, 1977; Hutchinson *et al*, 1981). Various reasons for this poor cytotoxicity have been suggested; inadequate cell numbers for analysis, low proportions of effector cells among TIL, inhibitory influences of tumour microenvironment (Miescher *et al*, 1986) or possible suppressive factors produced by cells in TIL populations (Vose *et al*, 1979). More recently Miescher *et al*, (1987) have found that cytolytic T lymphocyte precursors were present in tumours at higher frequencies than previously recorded. The tumours tested comprised melanoma, glioma, breast, colon and oesophageal carcinomas. They isolated TIL and assessed functional profiles by co-culturing TIL with disaggregated tumours. They pointed out that individual tumours had infiltrates with extremely heterogeneous functional profiles. Their study found a high number of TIL were T4+ cytotoxic T cells. This is a rare functional subset of normal peripheral blood T lymphocytes, which suggested that this type of cell was selectively accumulated *in situ*.

Boscia *et al*, (1988) studied TIL from human squamous cell carcinoma of tongue and found reduced cytotoxic function. They expanded TIL in IL-2 and found that this resulted in enriched populations of suppressor/cytotoxic T cells, compared to expanded peripheral blood lymphocytes (PBL) which were enriched with helper/inducer T cells.

These latter studies required the isolation of TIL from tumours and tested for cytotoxicity on autologous or allogeneic tumour cells. Functional studies *in vitro* may not be representative of TIL *in situ* in tumour, as they employ TIL and tumours which have been disaggregated and no longer retain the exact cell-cell interactions confined within tumour tissue architecture. Thus many mediators of T cell function may be different in the test situation compared to the *in vivo* situation. Furthermore, the isolation of TIL from solid tumour requires harsh separation techniques, such as those described by Zettergren *et al*, (1973), in which only small numbers of cells may be processed. Yields are often contaminated with tumour cells and lymphoid cell numbers are low. Present isolation techniques cannot provide healthy or representative tumour-infiltrating lymphocytes for functional and even, perhaps locomotion studies.

STUDIES OF CELL MIGRATION

Cell locomotion assays have contributed to our understanding of immunology and cancer. They can be classified as end-point and visual assays. End-point assays observe only the position of leading motile cells moving towards an attractant. The latter has been used frequently in the study of motile lymphocytes, but gives no information about the pathways or locomotion *per se*. Laryngeal carcinoma cultures were examined in this study by the visual assay method of time-lapse video microscopy. Visual assays allow more detail to be studied than end-point assays. They are used to investigate locomotory behaviour and interactions between different cells. Video technology for this was derived from closed circuit television security surveillance. During the early 1980s it was adapted for use with the light microscope and cell movement was recorded on video tape (Newgreen *et al*, 1982; Magee *et al*, 1987; Kuwana *et al*, 1987). Cell movement had previously been filmed only on reel to reel cinematographic celluloid film. This has the benefit of a more sharply defined image, but has the major drawback that there is a delay for developing before the image can be seen. This delay is considerable in terms of real time cell locomotion. Video on the other hand produces a slightly inferior quality picture to ciné film, but has the advantage that the image can be immediately replayed on the same equipment as for recording, without processing.

Locomotion of Cancer Cells

Cinematographic time-lapse observations of explant tumour culture have advanced knowledge of cellular growth and invasion. Although tissue culture cannot simulate the immense complexity of the conditions *in vivo*,

some of the distinctive features of malignant invasion have been conveniently observed by time-lapse cinematography. Abercrombie and Heaysman (1976) published an analysis of invasion *in vitro*. They used time-lapse film of fibroblasts and sarcoma cells derived from explants to demonstrate collisions between two cells in a two dimensional confrontation. A cell will cease to move in the same direction after contact with another cell. This is termed contact inhibition. The phenomenon of contact inhibition was found to be defective in tumour cells.

Similarly, the role of motility in invasive carcinoma has been studied in two-dimensional confrontations by Easty *et al*, (1982). They grew normal epithelial culture next to squamous carcinoma cell lines of the tongue and larynx. Normal cells were seen to move away from carcinoma cells. It could not be established whether this was important in invasion *in vivo*. Other confrontations of invasive cells and non-invasive cells were carried out in the form of mammary carcinoma cells seeded on endothelial cell monolayers (Zamora *et al*, 1980). This demonstrated that the system is a useful model for analysing the mechanism of tumour embolus interaction with blood vessel walls.

Locomotion of Lymphocytes

Part of the work carried out for this thesis is concerned with lymphocyte motility. Lymphocytes are intrinsically motile cells and their motility is a controlled process; if it were not they would be randomly dispersed throughout the body. Lymphocytes are constantly moving and repositioning themselves throughout the lymphoid system of thymus, spleen, bone marrow, lymph nodes, gut mucosa and other mucosal-associated tissues.

The ability to initiate an immune response depends upon the capacity of lymphocytes with appropriate receptors to move to an area where antigen is deposited, such as the skin or gut mucosa. This requires that lymphocytes must locomote actively out of the blood and cross into the lymph via high endothelial vessels. The subject of lymphocyte traffic and homing has been studied in intact animals, but gives little understanding of lymphocyte locomotion *per se*. However, the mechanism of lymphocyte locomotion has been derived from *in vitro* studies, which will be reviewed.

T lymphocytes motile on laryngeal cultures were shown to move towards undefined stimuli in the cultures. An investigation of the type of locomotion will be described in Chapter 7. The terminology of lymphocyte locomotion requires explanation and will be outlined here. Lymphocyte motility may be random locomotion, which is termed a *kinesis* or directed locomotion, which is termed a *taxis*. Various types of kinesis are known. These are *chemo*kinesis, where a chemical stimulus causes random locomotion; *ortho*kinesis, where speed is determined by the intensity of the stimulus, or *klino*kinesis where turning is determined by the intensity of the stimulus. Likewise, taxes may be prefixed according to the nature of the stimulus, although the most common type of taxis is *chemo*taxis. The most commonly used method for demonstrating chemotaxis of leucocytes by end-point assay (ie. measuring the lymphocytes nearest to the stimulus and therefore furthest from the starting point) is the Boyden filter assay (Boyden, 1962). A porous filter separates two compartments. In the upper compartment are cells; in the lower one an attractant. The attractant diffuses up through the pores of the filter, forming a gradient. The cells respond by migrating through the pores of the filter (which must be large enough to let this happen) towards the lower surface. Thus, movement of cells such as leucocytes to an attractant, can be measured. However, this

method provides only **indirect** evidence for chemotaxis. It does not demonstrate the features of morphological orientation and directional locomotion. More useful for lymphocyte studies are two-dimensional agar or three-dimensional collagen gel matrices, which are transparent and therefore lend themselves to visual assays of chemotaxis. Visual assays provide **direct** evidence of chemotaxis. Wilkinson, (1985) first used a direct method to show that lymphocytes move chemotactically in response to a stimulus using collagen gel as the substratum and time-lapse cinemicrography.

In vitro studies of lymphocyte chemotaxis and chemokinesis have shown that B and T lymphocytes respond differently to stimulants. Ward *et al*, (1977) found that T cells migrated to mixed lymphocyte culture supernatants but B cells did not. Locomotor capacity of human blood lymphocytes has been described by Wilkinson, (1986) and is linked to several parameters:

1) an increase in size of lymphocyte,

2) RNA and protein synthesis, but not DNA synthesis

3) a change from spherical to polarized shape

4) culture of lymphocytes in fetal calf serum for 72h

5) culture in the presence of mitogens, such as phytohaemagglutinin, polypeptide derivative, mixed lymphocyte culture or anti-T3 monoclonal antibody (OKT3). Lymphoblasts are also highly motile cells.

The earliest detailed studies of lymphocyte locomotion *in vitro* were made in the inter-war years. Lewis and Webster, (1921) watched lymphocytes moving out of explants of lymph nodes in plasma clot preparations *in vitro* and observed that the cells took irregular pathways and showed considerable variation in speed, the mean speed being

aproximately 10-20 μm/min. They described the polarised morphology adopted by moving cells with the nucleus positioned anteriorly and with an elongated tail containing cytoplasmic organelles. This was named the uropod. The uropod is important in the phenomenon known as peripolesis. Peripolesis is the process whereby lymphocytes are restricted in their locomotion to the surface of a tumour cell or macrophage (Sharp and Burwell, 1960). Humble *et al*, (1956) observed that lymphocytes tended to cluster around megakaryocytes in culture. The process of emperipolesis has been observed by the latter and several other groups. Emperipolesis describes a process in which lymphocytes enter other cells and move for long periods within their cytoplasm. Ciné films of this process have been made. Köller and Waymouth, (1953) observed leucocytes (mainly lymphocytes) emerging from explants of rat sarcoma. These leucocytes were seen to enter tumour cells *in vitro* and moved actively within the host cytoplasm. Pulvertaft (1959) presented a time-lapse ciné film of lymphocyte attraction to mitotic megaloblasts in culture, following stimulation of megaloblasts with folic acid. Humble *et al*, (1956) describe films showing lymphocytes entering malignant cells *in vitro*. Richters *et al*, (1964) described the behaviour (peripolesis and emperipolesis) of lymphocytes in primary explants of human lung cancers *in vitro*. Lewis, (1931) used time-lapse cinematography to study the movement of rat lymphocytes from lymph nodes which had been cultured for 24h *in vitro*. He describes morphology of moving lymphocytes. Characteristically, the cell puts forward a broad pseudopodium in the direction in which the cell is about to move. This shape change has recently been used in an assay of the response of leucocytes to locomotor stimulation by Haston and Shields, (1985).

Lymphocyte Locomotion in Collagen Gel

Collagen gel was used as a substratum for motile lymphocytes derived from laryngeal carcinoma explants. (See Chapter 5). In view of this the literature was searched for applications showing its use for demonstrating locomotion of normal and neoplastic lymphocytes. Migration studies have investigated the migration of neoplastic lymphocytes (Hogeman *et al*, 1987) and normal peripheral blood lymphocytes on collagen gels (Haston *et al*, 1982). These authors suggest that this type of gel is a good model for the microenvironment of the lymph node. Ratner *et al*, (1985) co-cultured adenocarcinoma cells with T lymphocytes in collagen gel matrices, which they purport to be a good model of the tumour microenvironment. However, migration studies in collagen may not adequately represent tissue environments, since lymphocytes move more readily in collagen than in contact with living substrata, such as monolayers of endothelium. The three-dimensional structure in collagen gels provides points of purchase, which lymphocytes use for leverage. No indication has been found that lymphocytes are stimulated by the collagen *per se* (Kubota, 1987). Although Sundqvist *et al*, (1986) report that T lymphocytes in contact with collagen become motile after 48h, but this motility is lost if the lymphocytes are transferred to a plastic substratum.

Heterotypic Co-culture Systems

Primary explant culture of laryngeal carcinomas provides a natural co-culture system of lymphocytes and tumour cells. The literature indicated that alternative studies have attempted to create artificial heterotypic co-culture systems with which to study lymphocyte migration. Chang *et al*, (1979) observed crawling movements of splenic lymphocytes

on 3T3 fibroblasts. This study found that most crawling lymphocytes are T cells, which move preferentially beneath but also along edges and on top of fibroblasts. Haston (1979) and Ager *et al*, (1988) have cultured splenic, lymph node and peripheral blood lymphocytes on cultured high endothelial cells (HEC). These lymphocytes adhere to and migrate beneath HEC, but also move over the monolayer. The finding in these studies that B cells adhere better than T cells is in marked contrast to the findings of Chang's group. Lymphocytes in general do not adhere to plastic substrata, thus preventing locomotion. These studies indicate that adhesion is necessary for the locomotion of lymphocytes in contact with HEC. Ager's group and Oppenheimer-Marks *et al*, (1988) have investigated whether this phenomenon is due solely to the structural presence of the cellular substratum, rather than any intrinsic biological factors. Lymphocyte adhesion was found to be independent of viable HEC, but migration was completely dependent on viable HEC.

Migration of Peripheral Blood Lymphocytes from Patients with Malignancy

Most studies of lymphocyte locomotion in cancer patients have assayed their PBL and assumed that their findings are relevant to the situation within carcinomas. Since there is a possibility that motile laryngeal lymphocytes are derived in part from the vasculature of the carcinoma, studies of PBL will be reviewed in this section. The literature regarding migration of immunological cells from peripheral blood of cancer patients focuses largely on T lymphocyte subsets. This is presumably because these cell infiltrates are most common in tumours. In lymphocyte investigations various workers have tried to understand the recruitment of lymphocytes to the tumour from the periphery. For this reason peripheral blood lymphocytes have been investigated more than tumour-infiltrating

lymphocytes. Comparative studies of peripheral blood lymphocytes from cancer patients and healthy controls are described by Hesse *et al*, (1984). Lymphocyte migration was found to be depressed in patients with established malignancy. This finding suggests that motility of lymphocytes derived from the vasculature of carcinoma explants may be impaired compared with that of lymphocytes derived from normal explants. Furthermore, responses to normal lymphocyte chemotactic stimulants, such as casein and Concanavalin A, were significantly reduced compared to those from normal controls and patients with benign tumour. They concluded that a factor may be produced by a population of lymphocytes in patients with a malignant tumour, which suppresses normal T cell migration. Similar findings are reported for head and neck cancer patients by Wanebo *et al*, (1978). Head and neck cancer was found to be unusual among carcinomas because cell-mediated immunity is also depressed at the premalignant stage (Pillai *et al*, 1987).

CHEMOTACTIC FACTORS

The finding in this thesis that lymphocytes are attracted chemotactically to mitotic cells in tumour and normal laryngeal cultures suggested that these cultures secrete soluble chemotactic factors. Therefore the literature was reviewed for reports of chemotactic factors produced by any of the cell types present in normal epithelium, mucosa and carcinomas. These included keratinocytes, carcinoma cells, fibroblasts, lymphocytes, monocytes, histiocytes, granulocytes, mast cells and plasma cells.

Delayed Hypersensitivity Skin Reaction

In vitro chemotaxis assay has identified chemotactic activity for lymphocytes in extracts of delayed hypersensitivity skin reaction sites (Cohen *et al*, 1973) and skin granuloma (Shimokawa *et al*, 1982) *inter alia*.

Mitogen-activated Lymphocytes

Culture supernatants from mitogen-activated lymphocytes are chemotactic for lymphocytes (Center *et al*, 1982). Chemotactic factors mediating the recruitment of lymphocytes to sites of antigenic challenge will be discussed, since the presence of neo-antigen induced by the growth of tumour may recruit lymphocytes to the tumour. The list of factors known to induce chemotaxis or chemokinesis has recently undergone dramatic expansion and continues to expand.

Inflammatory Skin Diseases and Mixed Skin Cell-Lymphocyte Reaction

Inflammatory skin diseases are characterised by infiltration of lymphocytes in the dermis as well as their invasion into the overlying epidermis. In the latter there is a close analogy to the situation in tumours. A model for this is the mixed skin cell-lymphocyte reaction described by Czernielewski (1985). This is the co-culture of dissociated epidermal cells with peripheral blood lymphocytes (PBL). PBL are stimulated to proliferate upon culture with epidermal cells of allogeneic and (to a lesser extent) autologous origin. This has been established by many groups (Morhenn *et al*, 1978; Braathen *et al*, 1980 and Czernielewski *et al*, 1982). The epidermal cells do not proliferate. This may be due to the fact that suspension of epithelial cells triggers the cells to differentiate terminally. (See above in section 'Tissue Culture by the Explant Method'.) This is a normal keratinocyte characteristic (also found among certain squamous cell carcinoma cell lines). This is one of the drawbacks of this model. Therefore the reaction is essentially comparable to a one-way mixed lymphocyte reaction (MLR). A comparable model for tumour cells is the mixed lymphocyte tumour culture (MLTC) This demonstrates T cell-mediated immunity to tumours (Rossio & Ruscetti, 1988). Unfortunately MLTC and MSLR are not realistic models for the activity of epidermal cells or carcinoma cells in response to lymphocytes *in vitro* or *in vivo*.

Epidermotropism and HLA-DR Antigen

The migration of lymphocytes into epidermis (epidermotropism) is effected by a variety of factors working individually or *in tandem*. HLA-DR antigen is one of the most potent biological signals. It is important in attracting and activating cytotoxic T lymphocytes. This is also called Ia antigen. It is expressed on Langerhans cells (Shiohara *et al*, 1988) and keratinocytes in inflammatory skin disease and skin graft rejection (Wikner *et al*, 1986). Cultured keratinocytes, including laryngeal squamous carcinoma cell lines, can be induced to express HLA-DR antigen by recombinant interferon-γ (rIFN-γ) (Koch *et al*, 1988). Furthermore HLA-DR expression may occur during cell maturation and differentiation (Nickoloff *et al*, 1986). Indeed some tumours naturally express HLA-DR antigen and may determine the capacity of tumours to stimulate lymphocytes, as demonstrated in the mixed lymphocyte-target cell interaction (MLTI) assay.

Interferon-γ

Keratinocytes treated with IFN-γ cause a three-fold increase in the proliferation of allogeneic lymphocytes (Morhenn *et al*, 1987). Lymphocyte proliferation can be chemotactic for other lymphocytes. Conflicting evidence has also been found to suggest that IFN-γ inhibits leucocyte proliferation in the presence of keratinocytes (Nickoloff *et al*, 1986) This is caused by the increase in prostaglandin E_2 (PGE_2) production from keratinocytes treated with IFN-γ.

Cytokines Produced by Keratinocytes

Other factors produced by keratinocytes, are chemotactic for lymphocytes. These are epidermal-thymocyte activating factor (ETAF), which is believed to be interleukin-1 (IL-1) (Lugar *et al*, 1982) and keratinocyte-derived T cell growth factor (KTGF), which is similar to IL-2, but functionally distinct (Kupper *et al*, 1986). IL-1 is produced by macrophages and is chemotactic for lymphocytes. This enables macrophages to present antigen to lymphocytes. Similarly keratinocytes may act as accessory antigen-presenting cells for lymphocytes. The production of strong immunological signals by keratinocytes suggested that they play a role in immunoregulation. Morhenn *et al*, (1987) investigated the possibility that HLA-DR positive keratinocytes become alloantigen-presenting cells for resting T cells and found this indeed to be the case.

Interleukin-2

Interleukin-2 (IL-2) is produced by T cells after mitogen or antigen-specific stimulation and has an immunoregulatory role including lymphocyte chemotaxis. IL-2 chemotaxis is specific for activated T cells (Kornfield *et al*, 1985).

Lymphocyte Activation

Activation of lymphocytes is necessary for locomotion and therefore is a requirement for a positive response to chemotactic factors. Wilkinson, (1986) has identified those previously mentioned effectors of

locomotion to be lymphocyte activating factors (i.e. phytohaemagglutinin (PHA), polypeptide derived factor (PPD), mixed lymphocyte culture and anti-T3 monoclonal antibody, which result in lymphocyte locomotion, by stimulating RNA synthesis and protein synthesis.

Fetal Calf Serum

My studies showed that fetal bovine or calf serum in the medium for the explant culture of laryngeal carcinoma was vital to successful culture, and that it was impossible to use serum-substituted medium. The stimulatory effect of serum on lymphocyte migration is indeed acknowledged. Ward *et al*, (1965) describe complement as a chemotactic agent for polymorphonuclear leucocytes (PMN). The heat inactivation of commercially available sera eradicates this effect to some extent. Boyden has shown there to be a heat labile factor in serum involved in PMN chemotaxis (Boyden, 1962), but is not clear if this is in fact complement. Serum is also stimulatory for lymphocytes. In the case of human lymphocytes there is considerable background stimulation (Johnson *et al*, 1965).

Lymphocyte Chemotactic Factor

Lymphocyte chemotactic factor (LCF) has been identified by several groups (Ward *et al*, 1977 and El-Nagger *et al*, 1982) to name but a few. LCF is produced from Concanavalin A-stimulated T lymphocytes, mainly by isolated suppressor/cytotoxic T cells and is a specific chemoattractant for helper/inducer T cells (Van Epps *et al*, 1983). Suppressor/cytotoxic T

are induced by antigen such as tetanus toxoid to produce LCF (Potter *et al*, 1986).

Summary of Chemotactic Factors

In summary of this section, lymphocyte-epidermal interactions show that the most important cell mediators of chemotaxis to inflammatory tissue are lymphocytes, macrophages and keratinocytes. Lymphocyte-epidermal interactions are complex and ill-understood. Unfortunately, many investigations of lymphocytes have been carried out on cells too remote from the situation they represent. Furthermore many studies have concentrated on mitogen- or antigen-dependent motility artificially induced *in vitro*. This probably cannot account for normal or pathological migration within tissues. The recent advances made in the field of carcinoma culture allied to the culture of tumour-infiltrating lymphocytes should allow more relevant studies to take place. It has emerged recently that keratinocytes can, themselves act as accessory immunological cells along-side lymphoid cells. Keratinocytes are now recognised as important cells in the skin immune system. Little is known about the role of carcinoma cells in this context, for want of more appropriate experimental models.

INHIBITORY FACTORS AFFECTING LYMPHOCYTE MOTILITY

The presence of migration inhibition factors within a tumour can have one of two possible effects. There may be an overall reduced recruitment to the tissue from the periphery resulting in a poor host response and failure to reject tumour. Conversely inhibition of locomotion may have a beneficial effect. If lymphocytes arriving in tumour tissue are immobilised, but can still proliferate, there may be an enhanced immunological reaction.

Defective T Lymphocyte Chemotactic Factor

Defective T lymphocyte chemotactic factor (LCF) production was found in patients with established malignancy. This was accompanied by impaired migration of patient T cells (Cole *et al*, 1986). The suppression of LCF production was caused by a circulating population of Leu 2 T-suppressor cells, not found in normal controls. What became clear from this work was that the production of lymphokines capable of stimulating T cell migration was affected by these suppressor cells. The authors speculated that this impairs tumour rejection.

Leucocyte Migration Inhibition Factor

A factor able to inhibit T cell migration is leucocyte migration inhibition factor (LMIF) This is a lymphokine produced by lymphocytes which restricts random leucocyte migration. Li *et al*, (1987) have shown that corticosteroids inhibit LMIF production, but antineoplastic agents do not. This may accumulate leucocytes. Therefore, there may be enhanced effectiveness of polymorphonuclear cells in acute inflammatory infections

including bacterial phagocytosis and killing. An additional effect of these agents was found by Li and co-workers. They suggest that hydrocortisone and certain anti-neoplastic agents (adriamycin and vincristine) affect production of IL-2 and gamma-interferon from peripheral blood mononuclear cells, but not their action if already formed. This may be important to the topic of this thesis because they are mediators of lymphocyte migration, although in the case of laryngeal carcinoma, chemotherapy or corticosteroids are not often given.

ADHESION OF LYMPHOCYTES

Importance of Adhesion in Lymphocyte Migration

It was necessary to review lymphocyte adhesion because many lymphocytes were dislodged from the culture during fixation. Furthermore the adhesion of lymphocytes is important for *in vitro* migration, as will be shown. The adhesive, locomotory and invasive properties of leucocytes have been known for over 100 years. Cohnheim's observations in 1882 were some of the earliest. Lymphocyte adhesion to non-haematopoietic cells is required for the induction and maintenance of immune responses and for effector function of lymphocytes. Chang *et al* , (1979) showed that spreading of lymphocytes on fibroblasts was associated with a high degree of motility and crawling between cells. Furthermore, there is evidence that lymphocytes, which in blood are nonadhesive, acquire adhesive properties in the vicinity of endothelium or other tissues they invade. Ford *et al*, (1978) found that nearly all agents which affect lymphocyte traffic also affect adhesion.

Functional Importance of Adhesion

Functionally, adhesion has been shown to be vital to lymphocyte recognition and destruction of tumour cells (Zucker-Franklin *et al*, 1983), and may determine infiltration into epidermis (Ternowitz *et al*, 1986). Lymphocyte adhesion to epithelial cells has also been investigated in patients with acquired immunodeficiency syndrome (AIDS) by Weber *et al* , (1986). Lymphocytes in AIDS usually possess multiple surface projections which indent and make point contact with adjacent epithelial cells. This has been purported to be important in immune injury in the gastrointestinal

tract. Close attachment and interaction between lymphocytes and laryngeal carcinoma cultures will be investigated in this thesis.

Lymphocyte Adhesion is Substratum-specific

Lymphocyte adhesion to cellular substrata was found to be substratum-specific by Ager *et al*, (1988). They showed that high endothelial cells in monolayer culture bound lymphocytes fifteen to two hundred-fold more efficiently than aortic endothelium, aortic fibroblasts or serum-coated glass. They also found that lymphocyte adhesion is absolutely dependent on viable lymphocytes, but independent of viable HEC.

Molecular Basis of Lymphocyte Adhesion

Currently there is very little literature regarding the molecular basis of T lymphocyte interactions and adhesion. The majority of such work has been carried out on fibroblast adhesion. Studies of epidermal cells have shown that mediators of lymphoblast adhesion to fibroblasts (tumour necrosis factor, bacterial lipopolysaccharide (LPS) and IL-1) are not equally effective in epidermal cell-lymphoblast adhesion. However, IFN-γ increases adhesion in both systems. Important molecules exist on epidermal cells treated with interferon-γ, which are ligands for lymphocyte function-associated antigen (LFA-1) expressed on lymphocytes. Ligand binding promotes adhesion and therefore migration. Rothlein *et al*, (1986) have identified a cell surface glycoprotein, intercellular adhesion molecule-1 (ICAM-1) present on sections of epidermal cells of the thymus and kidney tubule. This is the primary keratinocyte surface molecule that regulates adhesion between lymphoblasts and keratinocytes. Additionally, it was found on mucosal epithelial cells and keratinocytes at sites of immune

responses (Dustin *et al*, 1986). The expression of ICAM-1 occurs with similar distribution to HLA-DR antigen on epidermal keratinocytes, suggesting that they share a common regulation. This co-expression may be important in cutaneous immune responses and also infiltration of lymphocytes into tissue. Dustin's group (1988) has detected ICAM-1 expression on cultured keratinocytes treated with IFN-γ and also occasionally on untreated keratinocytes.

In an attempt to find comparative studies of lymphocyte adhesion and migration on monolayers, lymphocyte adhesion to fibroblast monolayers are included. The same ICAM-1 ligand is expressed on fibroblasts and therefore adherent lymphocytes should be comparable. Binding of lymphocytes to fibroblasts is dependent on characteristics of the lymphocytes, such as maturation. Poor binding is found among immature precursor lymphocytes, while mature lymphocytes adhere well (Abraham *et al*, 1988). Mitogenic stimulation of lymphocytes promotes adhesion (Kurki *et al*, 1987) as well as locomotion (Wilkinson, 1986).

THE PHENOTYPE AND FUNCTION OF MOTILE LYMPHOCYTES

In order to understand and interpret the responses of lymphocytes on laryngeal cultures it was necessary to review the literature of the functions of motile lymphocytes. Few systematic studies have been devoted to an understanding of the relationship between lymphocyte motility and lymphocyte function. Parrott and Wilkinson (1981) have reviewed the available knowledge up to 1981. More recently, monoclonal antibodies have allowed more precise lymphocyte phenotyping to be carried out. Migration studies carried out by several groups on stimulated T cells from peripheral blood and lymph nodes, have demonstrated that helper/inducer cells are more motile than suppressor/cytotoxic cells.

Different Locomotor Characteristics of Lymphocyte Subsets

Hoffman *et al*, (1983) found that subpopulations of lymphocytes demonstrate locomotor heterogeneity. They correlated the fastest moving lymphocytes with reduced cell-mediated cytotoxicity. These were identified as Lyt1+ (helper/inducer) T cells. In 1985, Ratner *et al* purported that T cell subset locomotion is differentially responsive to the types of microenvironmental conditions that exist in tumours. This was based on the motile cell population of lymphocytes derived from lymph nodes, in which was found a significantly increased ratio of Lyt1+ (helper/inducer T cells) : Lyt2+ (suppressor/cytotoxic T cells). Both these studies used murine lymphocytes and therefore used markers for murine antigens: Lyt1 and Lyt2. Potter *et al*, (1986) have demonstrated using human lymphocytes and markers for human antigens Leu-2 and Leu-3, that Leu-3 helper/inducer

T cells migrate chemotactically towards Leu-2 suppressor/cytotoxic T cells.

Importance of Lymphocyte Locomotion for Cytotoxic Function

Motility of cytotoxic T lymphocytes has been observed during lytic activity by many groups (Able *et al*, 1970; Sanderson, 1976 and Rothstein *et al*, 1978). Cerottini *et al*, (1972) and Plaut *et al*, (1973) found that cytochalasin B and colchicine inhibit lymphocyte locomotion and also block cytotoxic T cell lysis of target cells. This suggested that inhibition of locomotion blocked cytotoxic function.

In Vitro Locomotion of Gut Intraepithelial Lymphocytes

A population comprising mainly suppressor/cytotoxic T cells is found infiltrating gut mucosa. This is a non-circulating population of cells known as intraepithelial lymphocytes. Migration studies by Baca *et al*, (1987) reveal different migratory ability *in vivo* compared to cells *in vitro*. IEL are highly motile *in vitro* but are non-motile *in vivo*. The reason for this is unknown. However this inability to migrate *in vivo* may be the reason these cells cannot migrate into lymphoid tissue after injection into mice. This may explain the inability for these cells to induce a graft-versus-host response.

Tumour-infiltrating Lymphocytes

Isolated tumour-infiltrating lymphocytes (TIL) fail to respond to lectins (eg phytohaemagglutinin) in the normal way of lymphocytes isolated from non-tumour tissues (i.e. they do not proliferate) (Whiteside *et al*, 1986). Miescher *et al*, (1986) have shown that this is not just an effect of the separation procedure, but a characteristic of isolated TIL populations. The author speculates whether a similar problem exists for intraepithelial lymphocytes, which may possibly be present in laryngeal epithelium. The conclusion which may be drawn from both cases is that TIL behave differently to normal lymphocytes at least in their *in vitro* function and/or migration.

Large Granular Lymphocytes and Natural Killer Cells

Other migratory lymphocytes are described by Natuk *et al*, (1987). Murine large granular lymphocytes (LGL) and natural killer cells, which are non-major histocompatibility-restricted cytotoxic cells, become activated by viral infection and migrate towards human recombinant IL-2. The same study revealed chemotaxis of cytotoxic T lymphocytes/LGL. The immunomodulatory function of this would be to attract these cells to sites of inflammation.

Anti-tumour Function of Migratory T Lymphocytes

Anti-tumour function of migratory cytotoxic lymphocytes has been tested and compared to the non-motile form. Cytotoxic tumoricidal

function of lymphocytes has been found to be depressed in peripheral blood lymphocytes stimulated to become motile by interleukin-2 (IL-2) (Ratner and Heppner, 1988). This may be important to the immunotherapy of cancer with IL-2 stimulated tumoricidal lymphocytes. The results would suggest that anti-tumour cytotoxicity of infiltrating lymphocytes in solid tumour would be poor. It is unfortunate this study has yet to be carried out on tumour-infiltrating lymphocytes. However, a study by Boscia *et al*, (1988) has begun to look at *in vitro* IL-2 stimulated tumour-infiltrating lymphocytes isolated from carcinoma of the tongue. These TIL proliferated better than peripheral blood lymphocytes in IL-2, and furthermore the therapeutic potential of these cells is greater than that of unstimulated freshly isolated tumour-infiltrating lymphocytes. Wang *et al*, (1986) have shown that TIL naturally migrate out of explanted tumour tissue. These are highly motile when cocultured with tumour cells. No further literature regarding the locomotor capacity of TIL has been found.

CHAPTER 3

EXPLANT METHOD OF CULTURE

EXPLANT METHOD OF CULTURE

INTRODUCTION

Experimental Design

In the literature review in Chapter 2, it was pointed out that primary cultures have been grown by the explant method from only a small number of laryngeal carcinomas. At the start of my research there was no appropriate method of explant culture, which had been tested on a large number of carcinomas. It was not known whether human tumours could grow for an extended period of time *in vitro* and still maintain their *in vivo* properties. The experimental design was selected to achieve the following aims:

1) to establish an explant method of producing cultures of human laryngeal carcinomas, without the use of chemical or physical agonists,

2) to test this method on a significant number of laryngeal neoplasms,

3) to carry out a detailed investigation of monolayers of laryngeal carcinoma grown by this technique,

4) to investigate characteristics of the resultant cultures by time-lapse video microscopy.

There was a constraint imposed by the application of time-lapse video microscopy. This necessitated that cells were grown on a two-dimensional substratum, so as to keep the monolayer in focus. The use of three-dimensional matrices would have been inappropriate for this study because migrating cells would disappear from view as they moved beyond the focal limits of the vertical plane. A recent study by Freeman *et al*, (1986) did grow human tumours on three-dimensional collagen gel matrices. This

included carcinomas, but not laryngeal carcinomas. They investigated the explant method but did not apply time-lapse microscopy.

CULTURE METHOD

Dissection of Larynx

Freshly excised larynxes removed at laryngectomy for laryngeal carcinoma or accompanying pharyngectomy for hypopharyngeal carcinoma were transported promptly from operating theatre to tissue culture laboratory, immersed in transport fluid (5% penicillin and streptomycin in Dulbecco's Minimal Essential Medium, Flow Labs. Scotland). Each larynx was opened along the posterior wall by cutting in the midline from the inferior cricoid region to the superior thyrohyoid ligament. The ossified thyroid cartilage fractured longitudinally along the anterior midline in most larynxes when they were opened. Fresh transport fluid was poured liberally over the specimen to remove blood, mucus and commensal microbes. Foul-smelling gangrenous tumours were not prepared for culture.

Excision of Tumour and True Cord

Figure 3.1 shows a supraglottic carcinoma and the typical areas sampled for tissue culture. Material for explantation was cut from the most healthy-looking ulcerating or fungoid tumour mass ensuring that no necrotic or non-neoplastic tissue was included. Size of sample depended on tumour size. Since squamous cell carcinoma was derived from squamous epithelium such as that covering the true cords, normal true cord mucosa was taken as a control sample. This was stripped from the underlying laryngeal skeleton and generally measured 10mm x 3mm x 1mm. In the

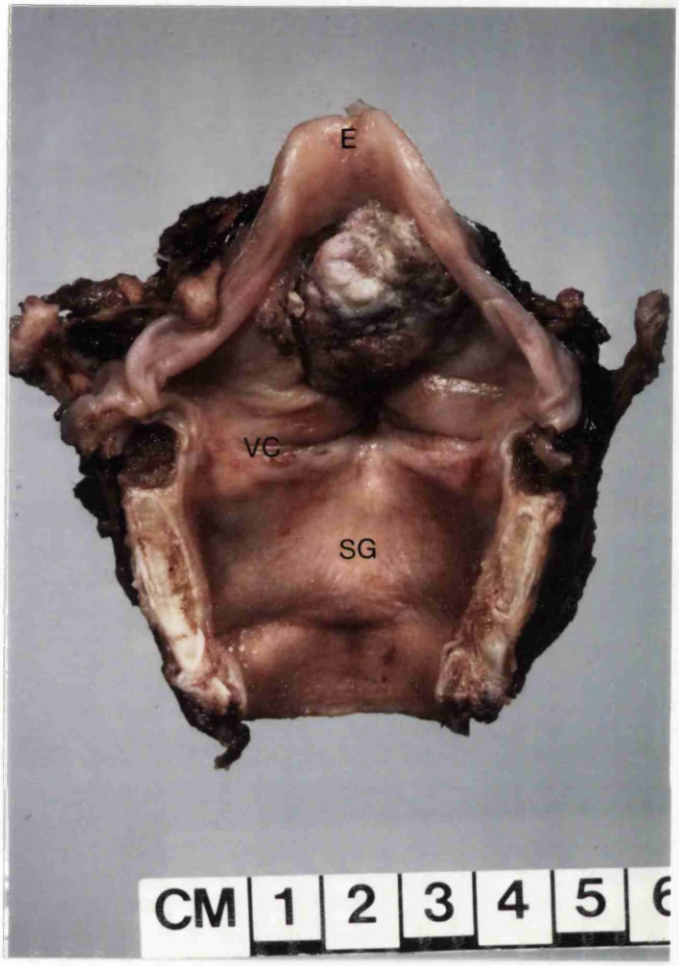


Fig 3.1 Human larynx opened posteriorly showing a squamous cell carcinoma projecting from the supraglottic region. This type of lesion was typical of those included in this study. Also shown are normal structures of the larynx. E = epiglottis; VC = vocal cords; SG = subglottis. In every case explants were also taken from normal true cord or other normal structures. Magnification x1.

absence of non-neoplastic tissue in this region an alternative site such as epiglottis or subglottis was selected and the mucosa sampled. Samples of tumour and normal mucosa for culture were washed in three changes of transport fluid to reduce microbial contamination, prior to culture. The number of explant preparations was dependent on sample size, numbering between twenty-five and 50. A maximum of three explants was placed on each coverslip. The main tumour and larynx were formalin-fixed and examined by the usual histopathological methods. A portion was also taken to be cryostat frozen sectioned for use in immunocytochemical studies. Any remaining tissue was permanently stored in liquid nitrogen.

Preparation of Explants

Any underlying connective tissue, white in contrast to the yellowish epithelium, was cut away with a scalpel, as a precaution against fibroblast proliferation. Control (normal true cord) specimens were flattened on a Petri dish with *stratum corneum* uppermost. Explants measuring 2mm x 2mm x 1mm were cut and placed on tissue culture grade poly-L-lysine coated plastic coverslips 10mm x 22mm (Flow Labs) with *stratum corneum* facing away from the lower substratum. Anchorage methods for epithelial tissues were investigated, such as allowing tissue to partially dry on to the substratum. This was not a strong enough anchorage for these explants. Embedding explants in plasma clots was avoided because the fibres of the clot would have prevented clear observation of tumour and epithelial cells. The method used in this study was a 'sandwich' technique; a coverslip was placed over the explants and joined to the lower coverslip by placing a hot dissection needle through both coverslips at opposite ends. Tumour was minced roughly with a scalpel into 2 mm^3 fragments. These were explanted as described for true cord. Coverslip 'sandwiches' containing explants were

anchored on the interior of plastic flasks (Flow Labs) by heat-affixing coverslip edges to the lower flask wall.

Serum Selection and Medium Preparation

Batches of test fetal calf serum (FCS) from a variety of suppliers (SeraLabs Ltd and Imperial Laboratories) were assessed for their ability to support growth of explants of normal forearm skin, when included as a supplement to the basic medium. The complete medium (described in detail below and in Appendix 1.3) comprised FCS, buffered DMEM containing phenol red pH colour indicator, glutamine and antibiotics (Flow Laboratories, Irvine, Scotland) Successful FCS batches were purchased in bulk (1 litre amounts) and frozen until use. Initially, culture medium was supplemented with epidermal growth factor (EGF), as used by Rheinwald and Green, (1977) and cholera toxin as used by Green, (1978) and hydrocortisone in place of a feeder layer. However, it was found that laryngeal explants could be cultured without EGF, cholera toxin or hydrocortisone, so these reagents were excluded from the medium for the culture of the majority of explants.

Medium was prepared by adding sterile preparations of glutamine (2% v/v), penicillin 100 IU/ml and streptomycin 100 µg/ml (1% v/v) and FCS (14% v/v) to DMEM at pH 7.4. The pH of the medium was checked before use, since fibroblast proliferation could be inhibited during the first 14 days by maintaining hydrogen ion concentration of growth medium at 7.4 (Parshley *et al*, 1950). This medium was called 'complete medium'. Complete medium was passed through a 0.22 µm pore-size cellulose acetate filter into flasks containing explants. A 10 ml volume was sufficient for a 50 ml flask, containing 3 pairs of coverslip.

Incubation

Explants were incubated in tightly closed flasks in a water-jacketed incubator at 36.5°C. Growing cultures were left undisturbed during the first 48 h.

Extra Precautions for the Investigation of Carcinoma Cultures

Carcinoma cells were extremely fragile and required careful handling. Precautions were taken such as maintaining a steady temperature on the microscope stage consistent with that of incubation, because some cultures were found to lose adhesion and died if the temperature varied.

Carcinomas were frequently infected. This created contamination problems unusual in a tissue culture laboratory. Reducing air-borne contaminants derived from infected explants by keeping flasks tightly closed, meant that the gaseous environment within the flasks could not be controlled. This did not appear to have an adverse effect on growth. Nevertheless it required that a check should be kept on the pH, since the buffering system relied upon a fine balance between air and CO_2 levels. If the medium became too alkaline, CO_2 was briefly fed into the flask. The pH did not often need to be controlled as the tissue and growing cells created a suitable gaseous environment, regulating the pH, during the short culture time of these experiments.

Failure to Grow

Each failure of explants to grow was carefully assessed in order to try to improve the method. One reason was incorrect positioning of tumour

explants, which unlike true cord, were irregular, so that the deeper side may not always have faced downwards. If tissue was heavily infected, the infecting organisms usually grew more rapidly than the tissue, so that the growth capacity of the tissue was unknown. However, several explants grew before a slow-growing mould within the explants killed the culture, which suggested that infected tissue may proliferate under these culture conditions. If all tumour and normal mucosa from the same larynx failed to grow, this was initially suspected to be a fault of the growth medium, and this possibility was excluded by changing the medium to one which was known could support growth. All growth medium was repeatedly tested for its ability to support the growth of explanted non-neoplastic epithelium, such as fetal or post-mortem tympanic membrane, adult forearm skin or neonatal foreskin. It was found to be necessary to assess continually the ability of medium to support the growth of epithelium. This meant that medium had to be prepared several days before it was required for culture of laryngeal tissues.

Immunocytochemical Anti-keratin Staining of Cultures and Sectioned Tissue

In this study the formation of intermediate filaments was investigated in specimens grown from 13 larynxes. Tumour and normal cultures, aged 7-14 d were stained with a panel of monoclonal antibodies (mabs) using the indirect peroxidase anti-peroxidase (PAP) technique and the chromagen diaminobenzoic acid, as described in Appendix 3.3. This panel of mabs comprised a broad range of anti-keratin mabs (Cam 5.2, PKK1, PKE, RGE 53 and RKSE 60) and an anti-mesenchymal marker: vimentin. These were used at dilutions given in Appendix 3.2. Cam 5.2 marks low molecular weight keratins. RKSE 60 marks high molecular weight keratins in suprabasal epidermal cells and keratinising cells of squamous carcinoma. RGE 53 marks

glandular epithelial cytokeratin 18, and PKK1 and PKE are general antikeratin markers.

Electron Microscopy of Laryngeal Cultures

Transmission electron microsopy has been found to be useful in the study of certain cancers. Both transmission and scanning electron microscopy have been widely used in dermatopathology. Details of intracellular organisation and cell surface structures are revealed respectively, and may provide insight into factors which distinguish normal tissue from pathological tissue. Transmission and scanning electron microscopy were carried out on normal and carcinomatous laryngeal cultures. Methods of processing are given in Appendix 3.1 and 3.1.1. Comparisons were made between normal and carcinoma cultures.

Time-lapse Video Microscopy of Laryngeal Cultures

Both normal and carcinoma cultures were recorded at intervals of 10 seconds using a Panasonic time-lapse VHS video recorder attached to a Nikon Diaphot phase-contrast inverted microscope. Such a method could be used to investigate the movements of the component parts of the culture as described by Boxall *et al*, (1988), or at higher objective powers the detailed events in individual cells in a culture (Dover *et al*, 1988).

RESULTS

Description of Explant Outgrowths

Primary cultures of laryngeal epithelium, like other epithelial cells in primary culture or cell lines, formed sheets, which were adherent on plastic coverslips. A cascade of epithelial cells emerged from the torn and exposed surface and dropped down the side of the explant onto the coverslip or adhered to the upper coverslip. Upon contact with the substratum, cells immediately became flat and adhered to the coverslip, spreading until movement ceased presumably as a result of 'contact inhibition' with adjacent cells. Monolayer sheets from normal and neoplastic explants were produced by the second day and thereafter until the 14th day.

Colony-like cell clusters with close-knit 'cobblestone' architecture emerged initially from one or more points on the explant edge (Fig. 3.2). Total epiboly (encasement of the explant by keratinocytes) did not occur. Outgrowth was restricted to particular arcs of the perimeter. At the points where culture was attached to the perimeter of the explant, the monolayer appeared stretched. There were two possible reasons for this:

a) the explant had shrunk during culture

b) the monolayer moved away from the explant, as proliferating cells enlarged the monolayer. This created 'attachment bridges' of extruded cytoplasm interspersed by holes where the coverslip was exposed, producing a trellis effect.

As cultures enlarged, the monolayer fanned outwards to surround other regions of non-proliferating explant, but were not directly connected to them, as they were at emerging points. Racking of the microscope objective

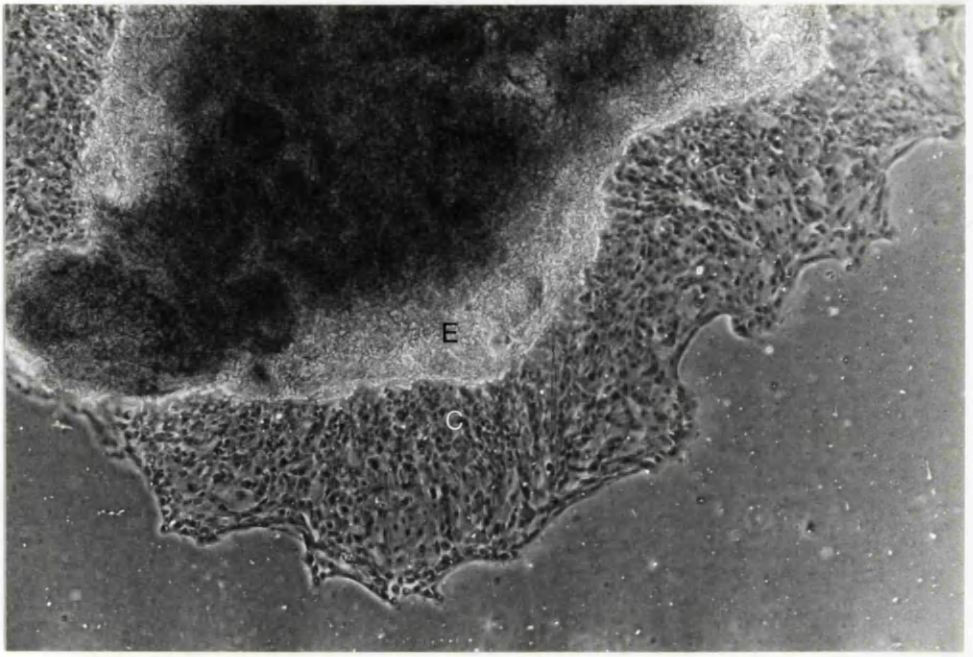


Fig 3.2 Tumour culture (C) at 4 days growing out from an explant (E). Culture is composed of typical epithelial cells. Magnification x146 phase-contrast.

revealed that culture descended from the explant edge onto the substratum, so that part of the monolayer closest to the explant was not in contact with the coverslip. A second monolayer of culture was often observed growing on the top coverslip. Light microscopy of early outgrowths (2-3 days) revealed outgrowths of uniformly small cuboidal cells with low nuclear to cytoplasmic ratios. Stratification was not observed by light microscopy. These cultures did not form a thick layer of squames similar to the *stratum corneum*.

Morphology of primary cultures became more heterogeneous than would be seen at any stage in the passage of established cell lines. After 7 d there were two distinct cell populations present in the monolayer. One population was composed of small cuboidal and the other of much larger cells, up to four times the area of the former, with greater irregularity and higher nuclear to cytoplasmic ratio. Several characteristics of the cultures resembled those of malignancy. Multinucleated cells, anisocytosis and mitoses were found in older tumour cultures, but were also present in older normal control cultures. All normal tissue was routinely confirmed to be free of tumour, and tumour confirmed to be squamous carcinoma. Towards the end of the proliferating phase (after approximately 25 d) the monolayer appeared to lose intercellular cohesion and adhered less well to the coverslip. During this phase granules appeared in the cytoplasm of the monolayer cells and the monolayer sloughed off the coverslip.

Confirmation of Growth

The emergence of an epithelial cell monolayer from one or more explants was recorded as positive growth. The yield of successful cultures from each case was between one and 20. Poor yields were frequent. The

main problem was that specimens were small, because most of the tissue was required for diagnostic purposes. Much of the tumour was often necrotic and only small amounts of non-necrotic tumour were available. There was some degree of contamination in many specimens, which further reduced the number of successfully grown cultures. The fragment of normal true cord submitted to culture was usually even smaller than that of tumour, but was less often infected. Table 3.1 gives a summary of cases which produced at least one successful culture and those which were complete failures. It can be seen that more tumours failed to grow or were lost due to contamination, than was the case with normal controls.

TABLE 3.1 **NUMBER OF CASES IN WHICH EXPLANT TISSUE GREW OR FAILED TO GROW**

	TUMOUR	NORMAL
CULTURED TISSUE	51	74
NO GROWTH	34	20
CONTAMINATED	20	11
TOTAL CASES	105	105

Further details of proliferative ability are presented in Appendix 2, Table 4

The Effect of Previous Radiotherapy on Explant Proliferative Ability

The literature did not reveal any investigations carried out on proliferation *in vitro* of preoperatively irradiated epithelial or carcinoma tissues. Dosage of irradiation varied slightly but the usual treatment was 5400-6000 cGy given in a course of 30 sessions over a period of 40-45 days. Irradiation treatment was often able to stop tumour growth in the early stages of laryngeal cancer. Since the neoplasms in this study had continued to grow, despite irradiation, it was interesting to enquire whether deep x-ray therapy (DXT) afforded tissue a better proliferative ability than untreated tissue and whether cultures differed in extent of growth or other characteristics. Alternatively the side effects caused by DXT, such as oedema and necrosis might have caused more contamination, thereby leading to more failed cultures.

Surprisingly there was not found to be any effect of irradiation on the ability of explants to proliferate. This is shown in Table 3.2 and presented for each larynx in Appendix 2, Table 8. The results show a similar success rate for DXT explants as seen in non-DXT explants. Eleven per cent non-DXT larynxes failed to grow both corresponding tumour and normal explants. The figure for DXT larynxes was lower at 3%. Likewise, if proliferation of tumour alone is considered, lower rates of failure are seen for DXT tumours at 23% than for non-DXT tumours at (31%).

No effect on morphology or keratinisation was found. Normal and tumour specimens from a larynx which had received both irradiation treatment and chemotherapy were also successfully cultured without appearing to be different from other preparations.

TABLE 3.2 **CASES OF NON-DXT AND DXT LARYNXES WHICH DID NOT GROW**

	NON-DXT	DXT
NORMAL & TUMOUR	8/70 (11%)	1/35 (3%)
TUMOUR ONLY	22/70 (31%)	8/35 (23%)

Contaminated Explants

Fungal and bacterial infection necessitated the termination of many explants before growth emerged, although fortunately there were usually several contaminant-free explants from many cases. The decision not to culture tumours which appeared or smelt as if they were contaminated was helpful in reducing the number of total failures. However, there were some tumours which appeared suitable, but which were later found to be contaminated. The numbers of cases of simultaneously contaminated tumour and corresponding contaminated normal tissue were similar in DXT and non-DXT specimens (17% of DXT and 20% of non-DXT specimens). A comparison with DXT cases indicated that failure due to contamination was equally common in both groups. Once an incubator became contaminated with fungal spores, it was a problem to eradicate the spores from the environment. The incubator could be disinfected adequately with formaldehyde but there was a strong risk that explants in open flasks might be poisoned if the incubator was poorly ventilated, following the procedure. As the spores were invariably derived from the tumours *per se*, it was decided to break from normal methodology and tightly seal the flasks so that no air-borne contaminant could enter or exit the flask.

Tumour Differentiation, Size and Growth of Culture

Histological differentiation, tumour diameter or the vertical extent of tumour in the larynx have been thought to be related to prognosis of laryngeal carcinoma. No correlation existed between these parameters and the ability to culture primary cultures from explanted tumour. There was no difference between the culture characteristics in cases with different

tumour grades (well, moderate or poor differentation) or vertical extension of tumour.

Variation in Cultures

The most striking difference was seen in the considerable variation of culture dimensions, even from different explants of the same biopsy. Some cultures were slow-growing and did not grow larger than the area of the explant, while others grew centrifugally to surround the explant with a 2-5mm width of monolayer. A small number of specimens produced excellent results from all explants. These were found to proliferate rapidly and extensively in every culture, and covered large areas up to 10mm, (the width of the coverslip). No explanation of this could be given; there was no correlation with histological or clinical data. Clearly some intrinsic property of the explant was responsible for unduly active proliferation characteristics. The growth factors provided in the fetal calf serum (FCS) did not appear to be important in this enhancement of growth, since results varied despite use of the same batch of FCS.

Tumour and Normal Cultures

Culture architecture was remarkably similar in tumour and normal epithelium (Fig 3.3). All cultures developed some morphological heterogeneity as referred to in other reports of explant cultures (see the discussion of this chapter). It might be suggested from this that the control laryngeal tissue was not free of tumour. Histological examination of sections from all tumours and normal tissue confirmed malignant neoplastic appearances in samples purported to come from tumour and normal appearances in samples purported to come from normal tissue however. Two

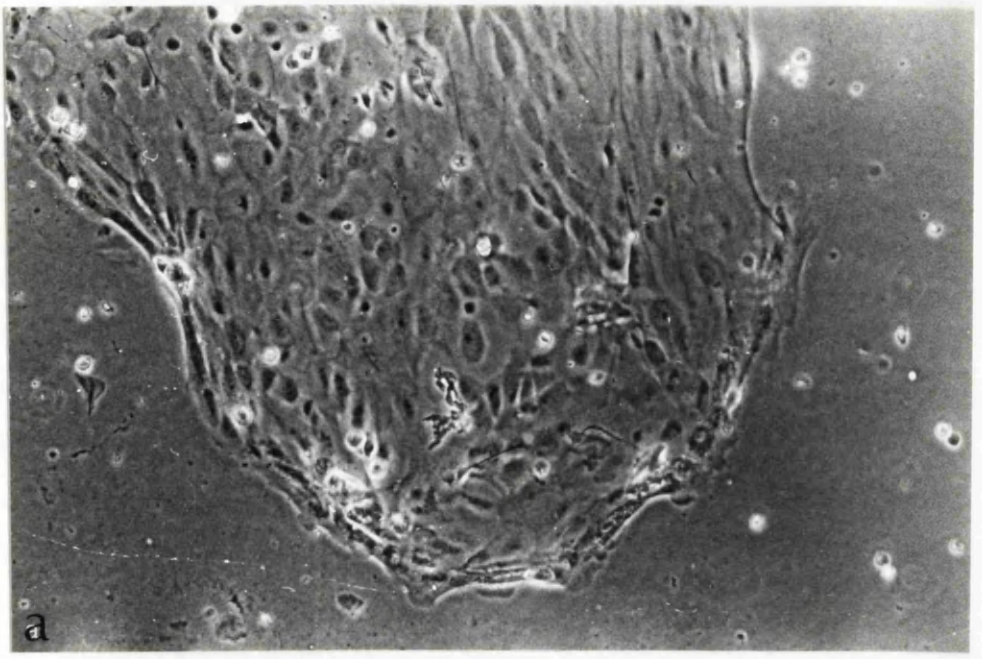
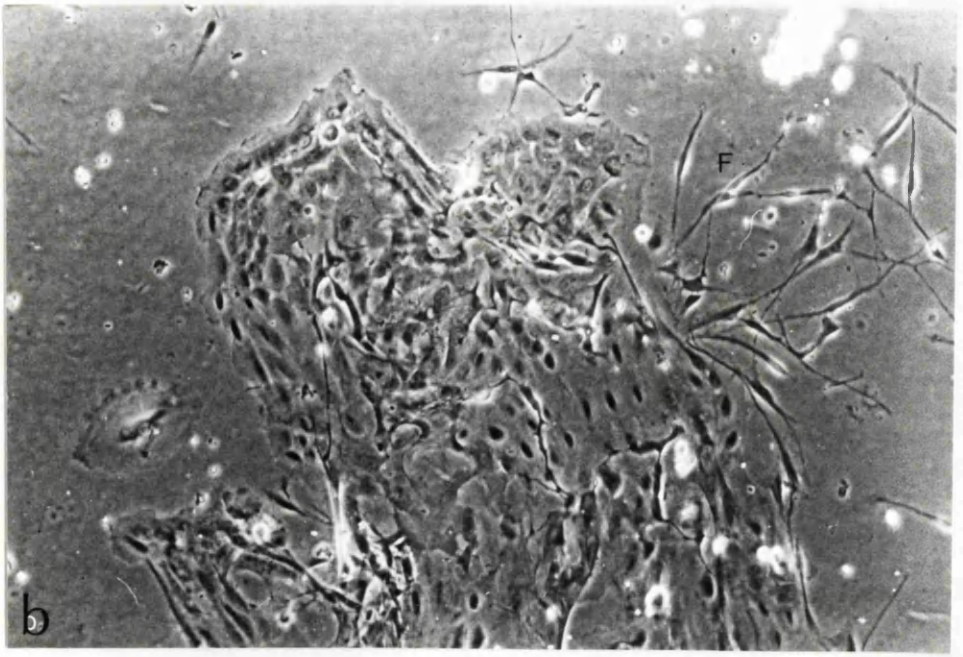


Fig 3.3 a) Tumour culture and b) non-neoplastic culture at 12 days. Morphology is similar in both cultures. A few fibroblasts (F) are present in b). Magnification x360 phase-contrast.



types of cell existed in discrete zones. Small cell type zones were present in the vicinity of the explant and scattered in foci throughout the monolayer and at the leading monolayer edge in both normal and tumour epithelia (Fig 3.4). Larger cell type zones surrounded the small cell foci in both types of culture, but were never close to the explant. The regularity of the large and small cells within discrete zones was a feature which was not found in bizarre cultures.

Bizarre Cultures

In 20% of cultures a random and bizarre appearance was observed in the monolayer (Fig 3.5). These cultures showed bodies resembling keratin pearls and more anisocytosis and multinucleated giant cells than other cultures. Bizarre morphology was observed in both tumour and normal cultures. No histological or clinical correlation was found. Time-lapse studies were performed and indicated that bizarre cultures are, in fact, non-proliferating (see Chapter 4).

Cytoplasmic Intermediate Filament Formation

There was no difference in staining reaction for tumours and control cultures. All cultures stained with PKK 1, Cam 5.2 and PKE. Figure 3.6 shows a culture stained with PKK 1. The stain was most dense close to the explant and became patchy until it diminished further away from the explant. Cultures were negative for RKSE 60 and RGE 53. Control sections of tumour and normal control were also stained. High molecular weight keratins were not present in sections of normal epithelium, but were found in tumour sections. Cultures and sections were found to stain with the

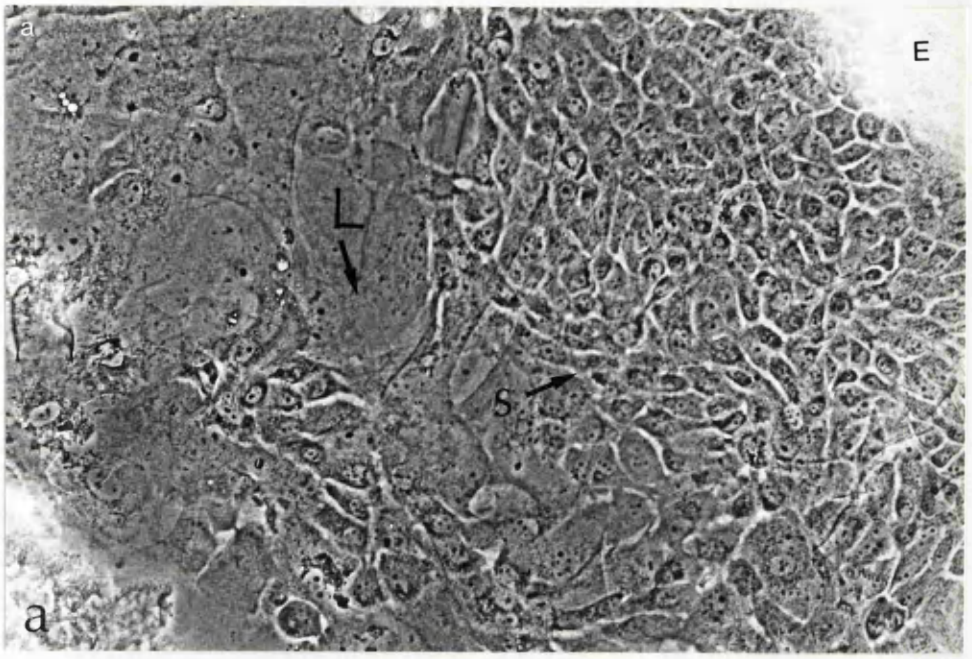
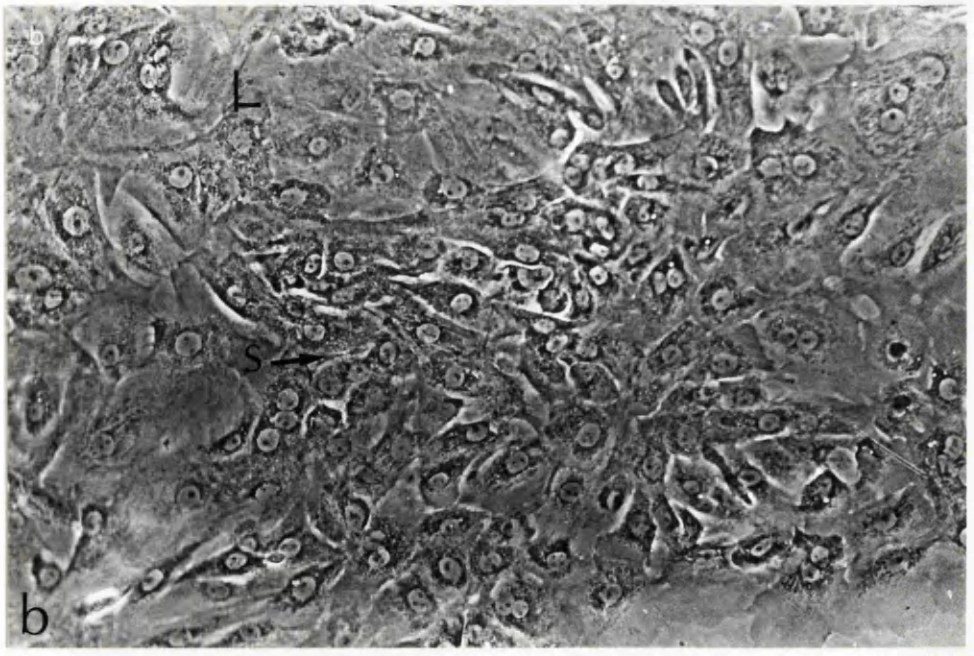


Fig 3.4 a) tumour culture and b) non-neoplastic culture at 12 days. Both cultures have areas of small cells (S) and areas of large cells (L). Magnification x360 phase-contrast.



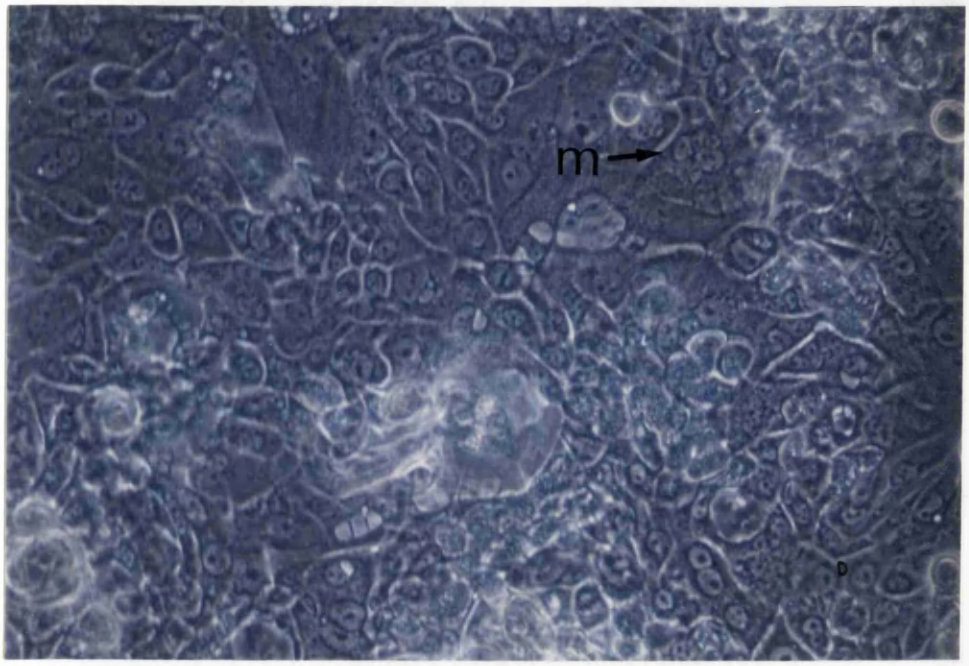


Fig 3.5 a) Bizarre morphology of tumour culture at 5 days. Anisocytosis and multinucleated cells (M) can be seen throughout the culture. Phase-contrast blue filter. Magnification x720.

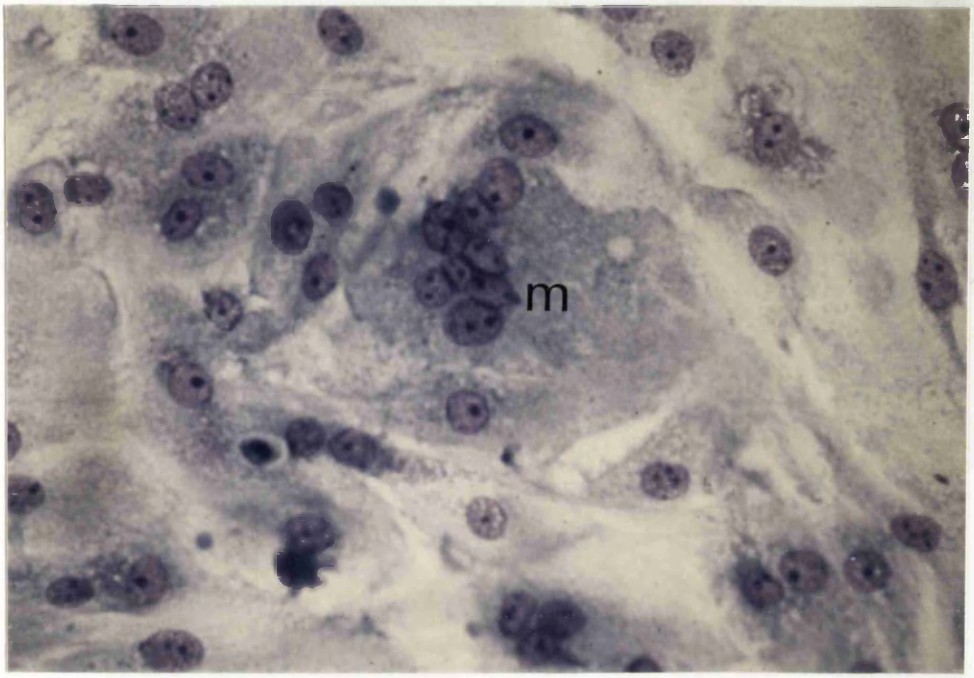


Fig 3.5 b) Bizarre culture of tumour at 5 days, showing a multinucleated cell (M). Leishman stain. Magnification x1440 oil immmersion.

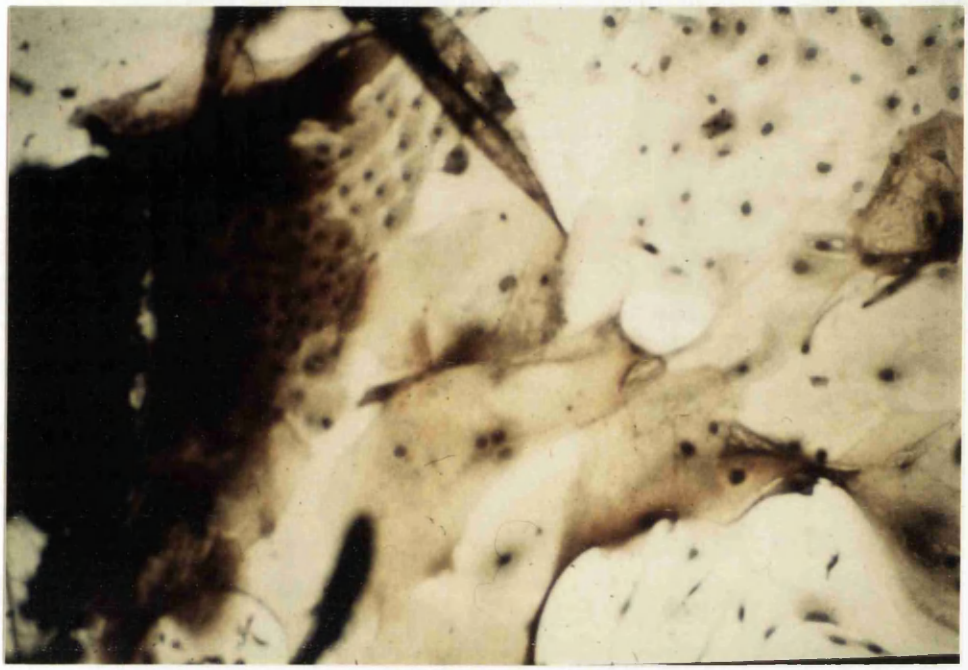


Fig 3.6 Tumour culture stained with PKK1 anti-cytokeratin monoclonal antibody using indirect PAP technique. Magnification x360

same anti-keratin monoclonal antibodies. Vimentin was not found in sections, although it was found in all cultures. The reason for this is unknown. Keratin formation was also confirmed using Ayoub-Schlar keratin stain (Fig 3.7).

Electron Microscopy

Transmission electron microscopy revealed that cultured carcinoma resembled normal epithelium. Tonofilaments and desmosomes in cultured tumour cells are shown in Figure 3.8. These were present in all cultured epithelial cells.

Scanning electron microscopy showed that individual cells have heterogeneous surface appearances. A microvillous surface was a characteristic of cultured epithelial cells, although the extent of this was variable. Some cells had hardly any microvilli as shown in Figure 3.9. Extremely diverse surface appearance was most pronounced in bizarre cultures. Examples of bizarre and other cultures are shown in Figure 3.10 a) and b) and Figure 3.11. Topographically, most non-bizarre cultures of tumour and control were found to be composed of expanses of flattened, tightly abutting cells adhering to the substratum. The monolayer was found to have gaps where the coverslip was exposed (Fig 3.11). Slight overlapping of cells also occurred (Fig 3.9), suggesting that random areas of culture were more than one cell thick. This was not recognised as stratification. There was some dome formation (Fig 3.12) and some areas in which cells had lost adherence to the substratum and become rounded (Fig 3.13). The latter cells were distinctly different from dome formation, although both retained a microvillous appearance, which is characteristic of epithelial cells. Round cells appeared phase-bright because as SEM has

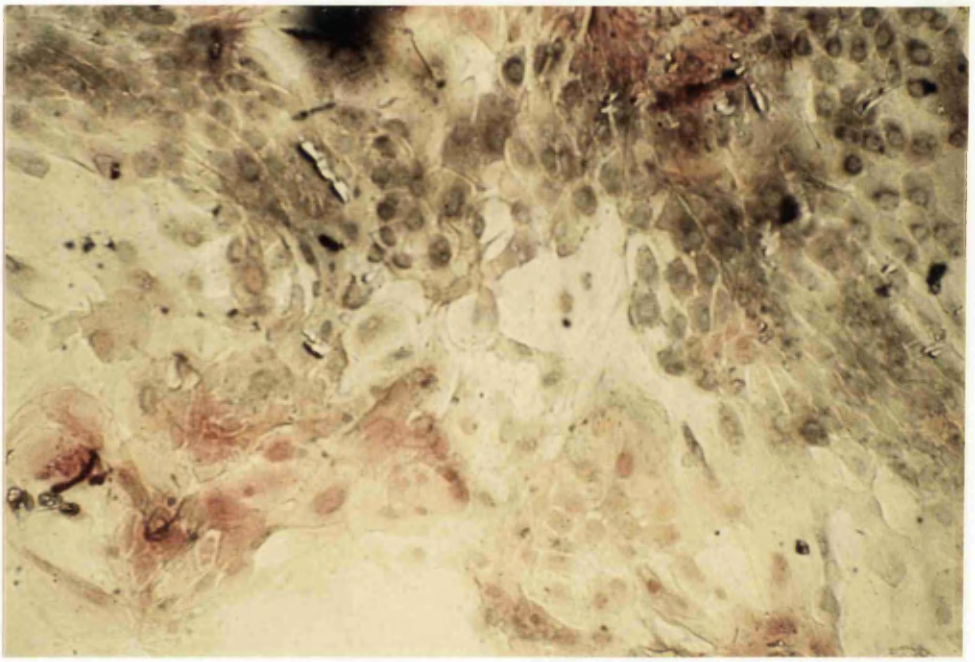
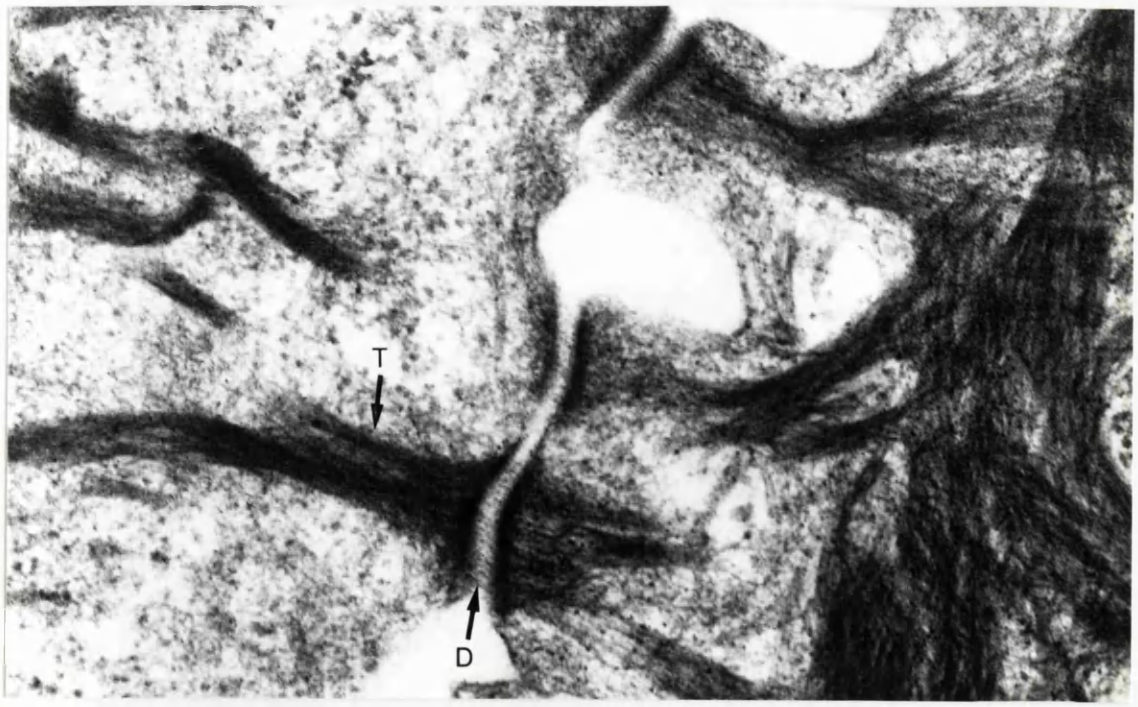


Fig 3.7 Ayoub-Schlar anti-keratin stain of non-neoplastic culture at 15 days. Red areas are rich in keratin. Magnification x323

Fig 3.8 a) Transmission electron micrograph of tumour culture. This cell has typical characteristics of an epithelial cell: desmosomes (D) and tonofilaments (T). Magnification x25568

Fig 3.8 b) Transmission electron micrograph of tumour culture showing desmosomes (D) and tonofilaments (T). Magnification x50795.



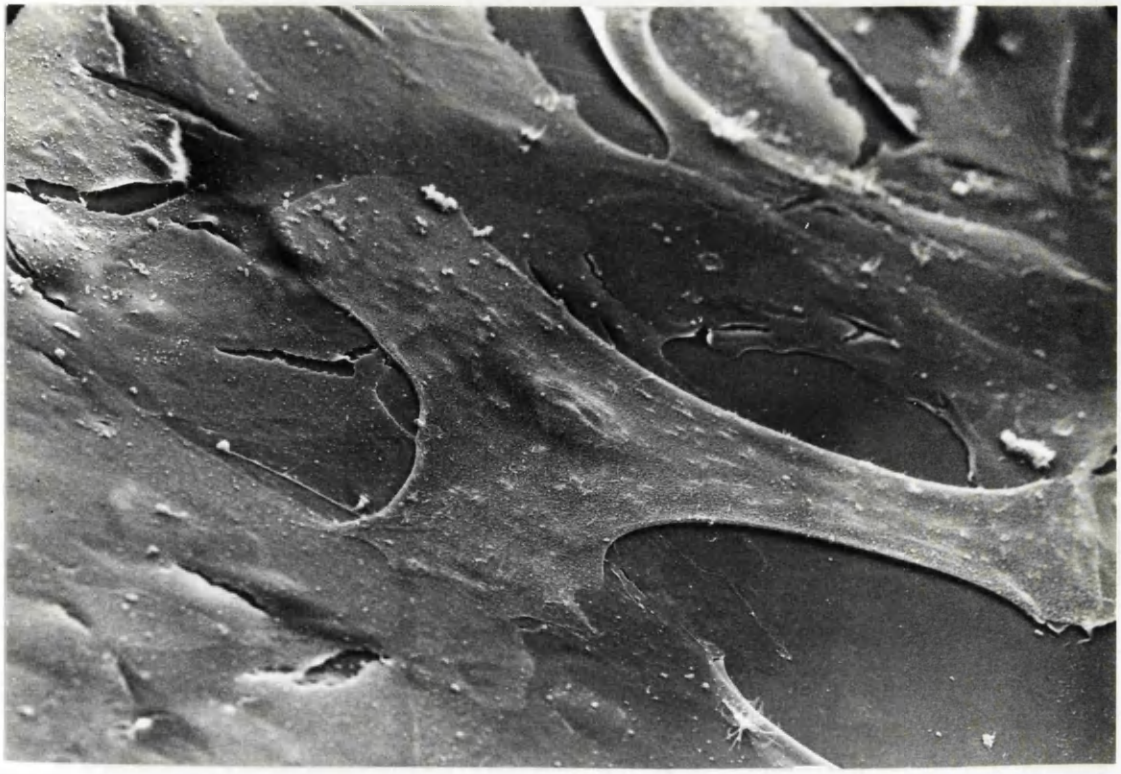


Fig 3.9 Scanning electron micrograph of non-neoplastic culture. Overlapping of cells is visible. Magnification x512.

Fig 3.10 a) & b) Scanning electron micrographs of bizarre tumour cultures. Magnification x1666.

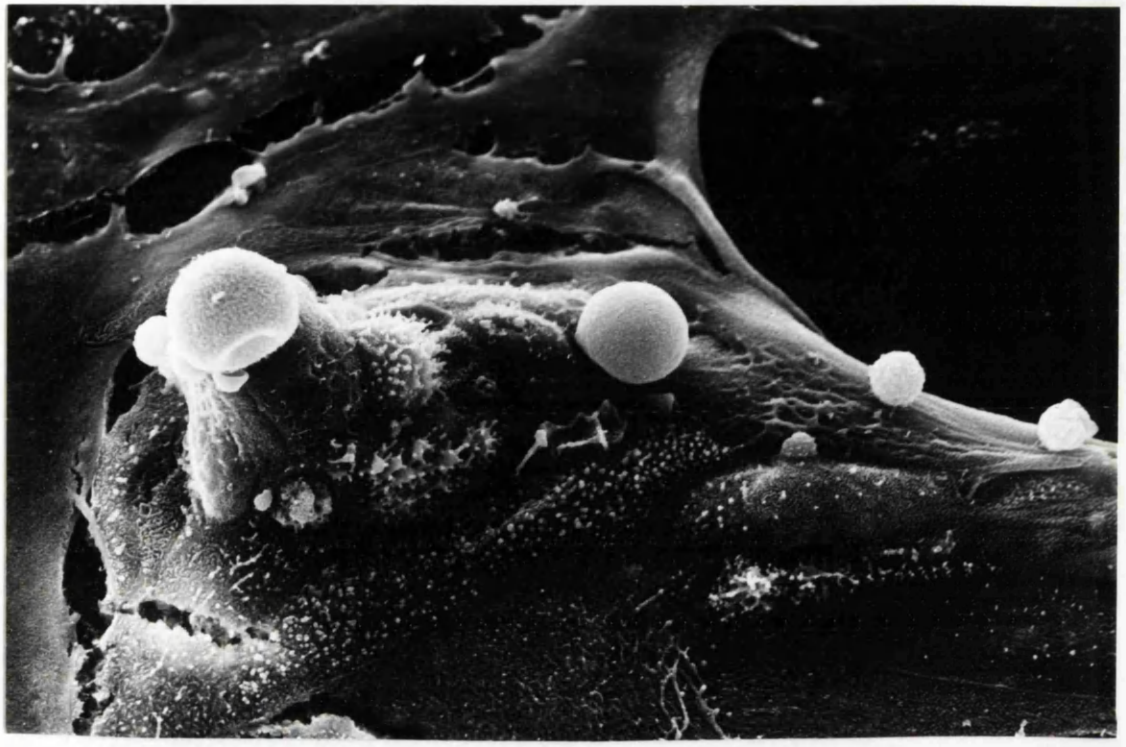


Fig 3.11 Scanning electron micrograph of non-bizarre tumour culture. Gaps in the monolayer, which expose the coverslip can be seen. Magnification x4519

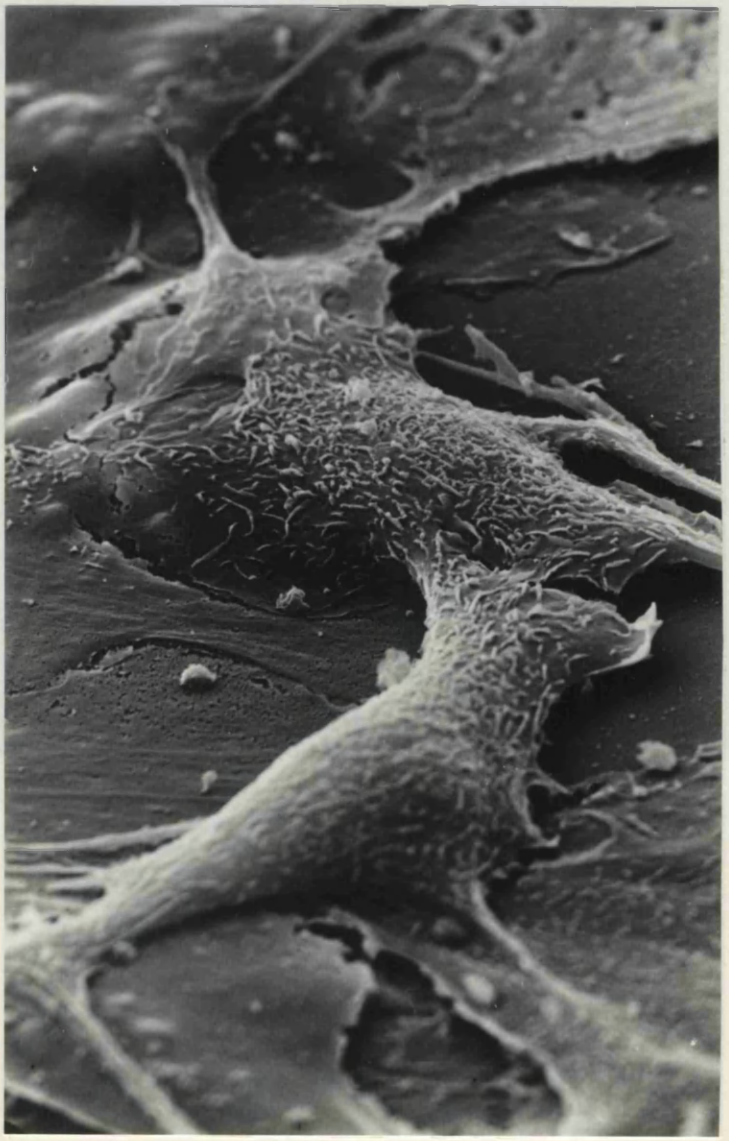


Fig 3.12 Scanning electron micrograph of tumour culture showing a domed cell. This suggests that this is a monolayer culture. Magnification x4903.

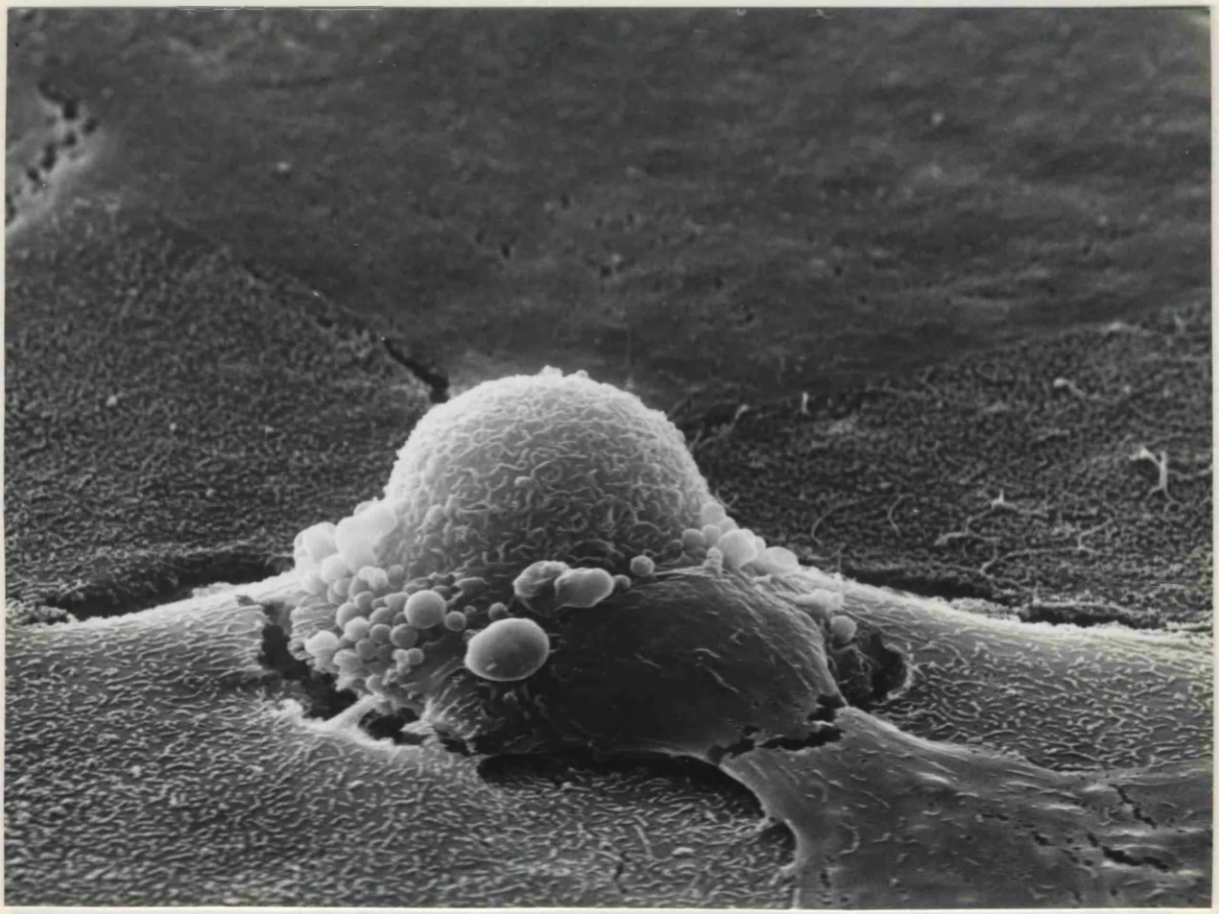
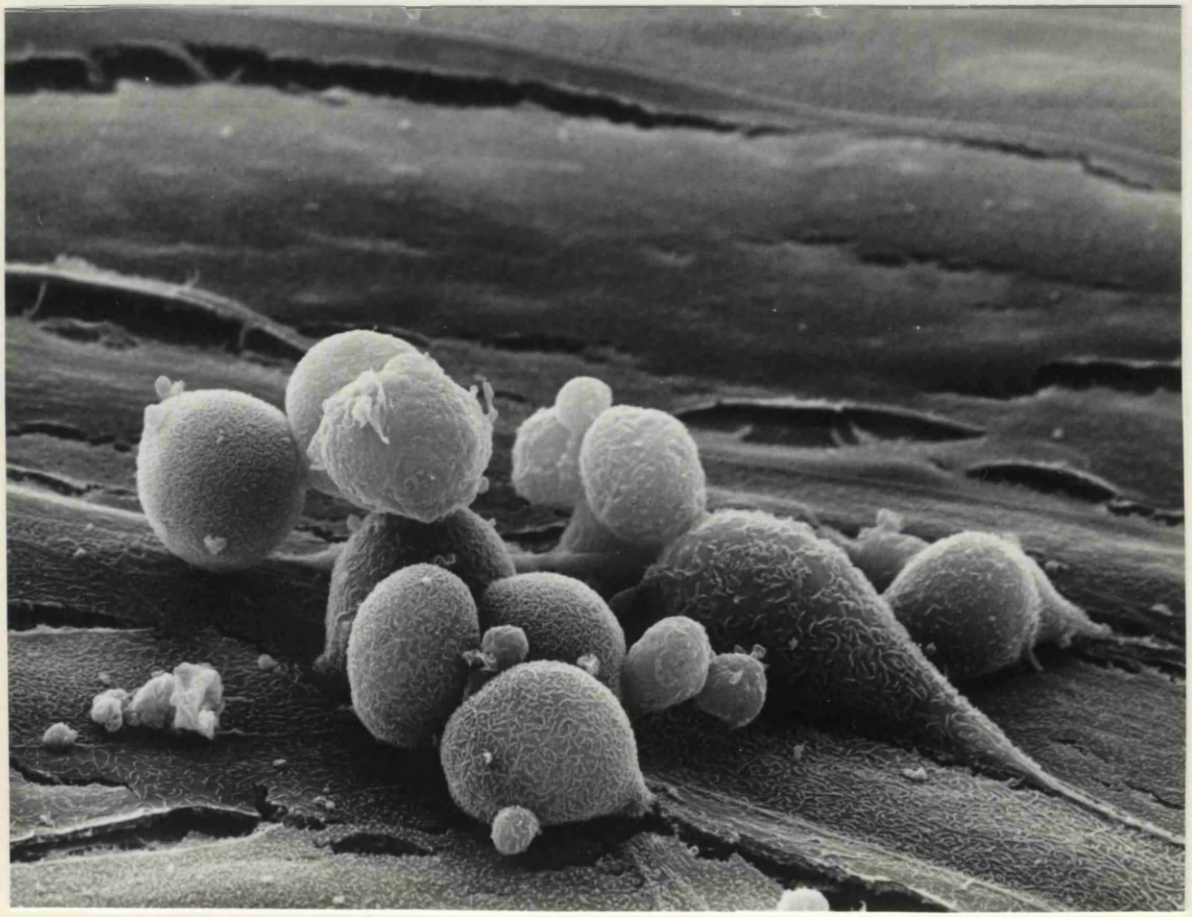


Fig 3.13 Scanning electron micrograph of tumour culture showing rounded epithelial cells. Magnification x2742



shown they are raised above the monolayer. These were non-motile. Domes were phase-dark (less raised) and observed best by SEM. The finding of domes (hemicysts) in laryngeal cultures is an indication that cultures are confluent. Domes are found if culture has been grown on an impermeable substratum. Domes have been associated with tight junctions in epithelial cell monolayers and are caused by a trapping of osmotically active molecules by circumferential tight junctions. The presence of tight junctions was confirmed in laryngeal cultures by TEM (Fig 3.8). Further evidence suggests that cells forming domes are mitotic, as described by Mullin *et al*, (1988). I found that mitotic cells in laryngeal cultures were domed. It was necessary to rack the objective between two depths of field, in order to show that the mitotic cell was raised above the surrounding cells.

Time-Lapse Video Microscopy

Cultures did not move 'en masse', unlike the migratory epithelium of the tympanic membrane and cholesteatoma (Boxall *et al*, 1988) although some single cells were seen to detach from the monolayer edge and moved independently on the coverslip substratum only. Reattachment to the monolayer was seen. Mitosis was seen in many cultures, except those which had a bizarre morphology. Mitosis created pressure zones forcing a gradual change in the morphology of the monolayer.

A motile population of cells was observed. These cells moved in an amoeboid fashion, which was unlike the amoeboid locomotion of granulocytes and monocytes on plastic substrata, being in contact with the monolayer and were likened to the wandering lymphocytes described by Marshall *et al*, (1965). These were identified after Leishman staining to have a similar

morphology to lymphocytes, although the appearance was rather flattened. The possibility that there might be other motile cells besides lymphocytes was contemplated, because the morphology was unusual (Fig 3.14). More cytoplasm was seen in motile lymphocytes than usually observed in mononuclear cells in blood smears. Motile leucocytes comprise two groups: polymorphonuclear neutrophils (PMN) and mononuclear leucocytes. The former do not remain viable in culture for more than 48h and therefore were unlikely to be detected in these laryngeal cultures. Mononuclear leucocytes lived longer in culture. They comprised monocytes and lymphocytes. These two cell types can be distinguished morphologically following staining. Monocytes and macrophages are identified by horse-shoe shaped nuclei and a higher nuclear cytoplasmic ratio than lymphocytes. Lymphocytes were identified by a large nucleus almost filling the entire cell. Motile lymphocytes also are known to have a characteristic motile shape. A tail forms and trails behind the main body which produces a leading lamella of one or two broad pseudopodia. Thus the shape resembles a hand-mirror, with the tail or uropod forming the handle. Motile cells seen on cultures were frequently of the hand-mirror morphology and in a preliminary study in which cultures were stained with Leishman, all were typical lymphocytes.

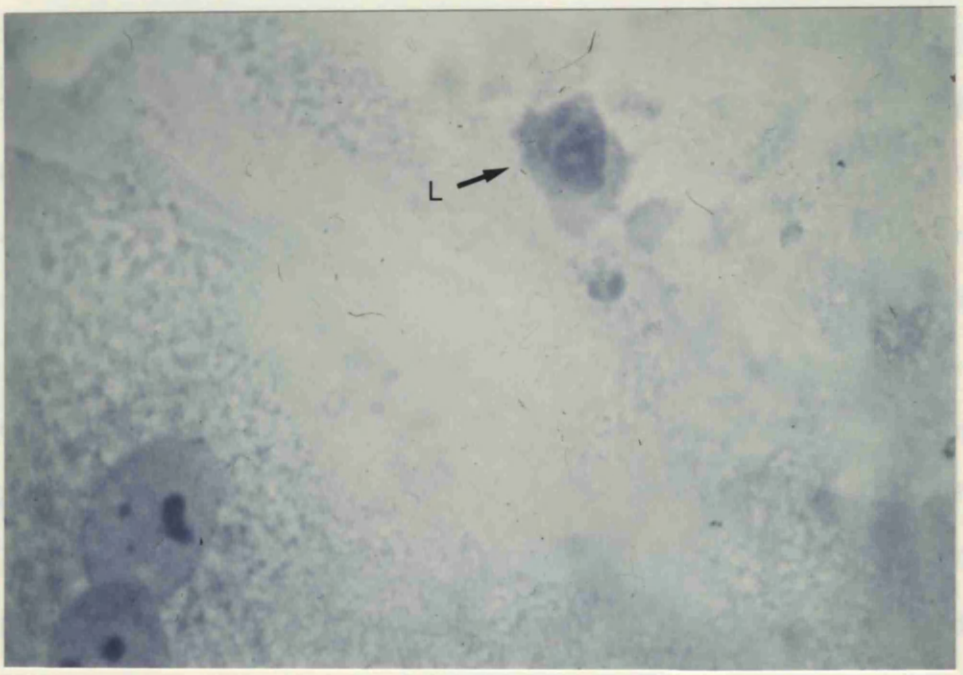


Fig 3.14 Leishman stained tumour culture with motile lymphocyte (L), which is rather flattened. Magnification x3600 oil immersion.

DISCUSSION

Epidermal Growth Factors in the Medium

The medium used to grow the majority of cultures comprised a physiological saline solution containing amino acids, undefined growth factors from serum, glutamine and antibiotics. The medium was buffered to maintain a constant pH. The need for epidermal growth factor was recognised by Tsao *et al*, (1982) to be necessary for keratinocyte proliferation in the absence of a feeder layer support system. I have found in these experiments that growth proceeded in the absence of epidermal growth factor (EGF) **and** feeder layer. This was fortunate since it has been reported that EGF can itself produce abnormal culture morphology of carcinomatous cells (Chinkers *et al*, 1979). Abnormal effects of EGF on human carcinoma cell line A-431 included the formation of multilayered colonies and extensive ruffling of the monolayer surface created by lamellipodia and filopodia. Other reports show that EGF stimulates pinocytosis in whole cells (Haigler *et al*, 1979) and protein phosphorylation in isolated cell membranes of A-431 cells (Carpenter *et al*, 1979). A feeder layer was avoided because it might produce undefined metabolic products which could have strong influences on explant cultures.

Alternative Growth Factors

The inclusion of cholera toxin and hydrocortisone as growth supplements was found to be unnecessary. The presence of fetal calf serum (FCS) and possibly the explant *per se* provided an undefined selection of growth factors in the culture environment. This method is dependent upon careful selection of growth-producing fetal calf serum. Batches of fetal

calf serum (FCS) from small numbers of animals were tested before use to assess the ability of each batch to support growth of epithelial cells. Only certain batches were able to support growth. Undefined growth factors are present in FCS. Platelet-derived growth factor and complement may be responsible for growth. In each test the optimum batch was found to be fresh fetal calf or bovine serum. It is advisable to purchase bulk amounts of the same batch. The importance of this cannot be overstressed. The possibility that growth factors cause abnormal results is thus restricted to the minimum by this simple approach. An increase in the concentration of fetal calf serum (FCS) as a means to providing more growth factor is not advisable since epithelial cells prefer low levels of FCS (10-15%). Levels of 20% FCS will inhibit proliferation.

The literature was searched for reports regarding the influence of FCS and explant tissue on keratinocyte proliferation. It was found that a synergistic response occurred if a mammalian peptide called Substance P was presented to keratinocytes in conjunction with FCS (Tanaka *et al*, 1988). Substance P is known to be present in inflammatory skin and to act as a mitogen for connective tissue. If present in epidermoid tumours Substance P could directly stimulate keratinocyte growth. It is not known if tumours produce this substance. Also, if the connective tissue in the explant were affected by this substance there might be a mechanism for stimulation of keratinocytes indirectly via growth factors produced naturally by non-proliferating fibroblasts, analogous to the situation existing if a feeder layer were present. This may explain the ability of explanted carcinomas and epithelial cells to grow without extra growth factors. Further support for such a mechanism in this system lies in the finding that serum-substitutes in medium from which serum was omitted failed to support growth of these explants.

Control of Fibroblast Outgrowth

Explants of epithelium and carcinoma were never free of a small amount of connective tissue, as trypsin was not used to separate the latter from the epithelium or carcinoma. Nevertheless, laryngeal cultures grew well without fibroblast outgrowth, under controlled conditions. This technique was reported to have similar success in growing epithelial cells free of fibroblasts by several other groups (Bassett *et al*, 1955 and Hsu 1952). Parshley *et al*, (1950) found that epithelial cells were highly sensitive to hydrogen ion concentration. Fibroblast proliferation was inhibited during the first 14 days in culture by careful control of the pH. At pH 7.8 epithelial cells did not proliferate but at pH 7.4 epithelial cells grew, while fibroblasts were inhibited. The simplicity of this method of fibroblast control suited this project because it required no harsh chemical or physical treatment.

Possible Advantages Derived from Fibroblasts

A small amount of fibroblast proliferation usually began after 14 d in all cultures. Thus fibroblasts in the explant remain viable during this period. It is interesting to consider the possible advantages of viable connective tissue cells in this culture system. The literature review has shown that other authors have found that dermal cells present in explants exert influences on epidermal carcinogenesis (Pinto *et al*, 1968) but further factors may be responsible for producing the correct growth conditions. There may be growth factors produced from non-proliferating fibroblasts in the explant, which in a local way are able to act as a feeder layer.

Keratinisation

Maturation of keratinocytes is usually associated with a switch in synthesis from small to large keratin molecules *in vivo*. This difference was observed between normal epithelium and dysplastic epithelium in sections of laryngeal tissue. High molecular weight intermediate filaments are restricted to dysplastic tissue. However, in culture the switch to large keratins does not occur. This agrees with the findings in skin epithelial cultures reported by Fuchs *et al*, (1980). Thus most cultures were negative for RKSE 60 which usually reacts with suprabasal epidermal cells and keratinising cells in squamous cell carcinomas in sections.

Characteristics of Culture

Culture appearance was similar in controls to that of tumour cultures. There was, however considerable heterogeneity of cell size and shape between cultures. This has been noticed in other studies of skin explant cultures by Resau *et al*, (1988). The larger cells did not mitose as frequently as smaller cells. Small cell zones were frequently seen to be mitotic. There was a degree of synchrony observed between neighbouring mitotic cells, which were identified as sister siblings. This has been reported in keratinocytes of neonatal foreskin by Dover *et al*, (1988). In laryngeal cultures synchronous mitoses were observed mainly in small cell zones.

Electron Microscopy

Tight junctions are responsible for retraction fibres which link mitotic cells to neighbouring cells, as seen in laryngeal primary cultures. This has been reported to occur in HEp-2 cultures by Harris, (1973).

Time-lapse Video Microscopy

Time-lapse video microscopy has shown three characteristics of primary explant laryngeal cultures:

1) that mitoses occur in the horizontal plane of the monolayer and the stages of anaphase and telophase are most prominent.

2) With the exception of some movement created by expanding cell numbers, very little movement of culture cell sheets was observed. This was thought to be due to the presence of explant tissue in contact with culture, forming an anchor. Some isolated tumour cells moved independently on the coverslip and over areas of culture. These were not common and therefore were not investigated.

3) Lymphocytic wandering in normal and tumour cultures was frequently seen and will be studied in more detail in subsequent chapters.

CHAPTER 4

LYMPHOCYTE MOTILITY

LYMPHOCYTE MOTILITY

INTRODUCTION

The aim of this chapter is to present the basic features regarding lymphocytes moving on cultures, as observed by time-lapse video microscopy. Also investigated was whether motile lymphocytes were restricted to certain cultures only.

METHOD

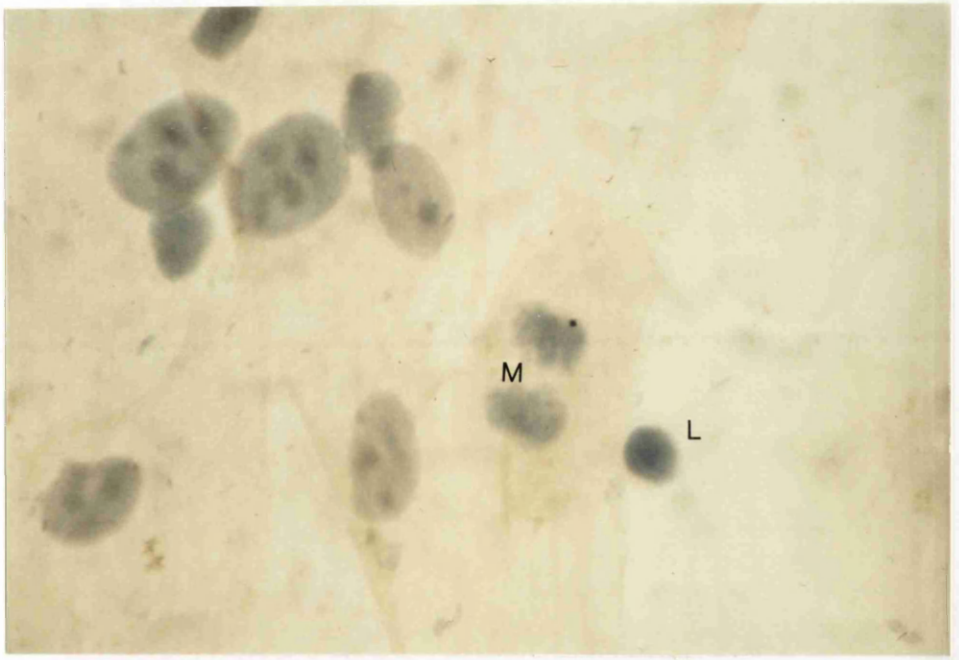
Cultures from a total of 38 control specimens and 35 tumours were studied using a x10 objective and phase-contrast inverted light microscope. Time-lapse video recording of cultures, aged between 2 d and 24 d, was carried out at a lapse interval of 10 seconds. Several sequences of different, arbitrary fields of each culture were recorded for a period of 24-48 h. Where possible autologous tumour and normal control cultures were both studied. However in some cases tumour culture was not available. Lymphocyte locomotion was easily detected by replaying tapes at normal speed. Comparisons were made between cultures which had motile lymphocytes and cultures which did not.

RESULTS

Of 38 non-neoplastic cultures, 30 were found to have motile lymphocytes. A similar proportion of tumour cultures had motile lymphocytes (27 out of 35). In all positive cultures between 2 d and 24 d, lymphocytes appeared phase-dark and moved in an amoeboid fashion, often following the route of cell borders in the substratum. Morphology of moving lymphocytes was characteristically polarised, with a leading lamella, tapering body and contraction waves which moved down the cell from front to back. A posterior uropod was frequently observed (see video at time code 00:03:35). Speeds varied between 6 μm/min and 24 μm/min. Bizarre cultures were rarely found to have motile lymphocytes and were not observed to have mitotic figures. Furthermore bizarre cultures ceased to demonstrate the usual signs of proliferation seen in other cultures, such as an active streaming of the cytoplasm and changing appearance of outgrowth. Many non-bizarre cultures often had motile lymphocytes and were frequently found to have mitotic figures. Seventy-five per cent of normal cultures and 89% of tumour cultures of irradiated larynxes were found to have motile lymphocytes. A similar proportion was found for cultures from non-irradiated larynxes (83% normal cultures and 74% tumour cultures).

Attraction of lymphocytes to mitotic cells of tumour and normal culture was observed in a small number of non-irradiated cultures only (Fig 4.1). The fact that the lymphocyte had a polarised morphology and made contact with a mitotic cell during the migration, suggested that mitotic figures could cause directed lymphocyte locomotion and be responsible for the phenomenon of attraction. Furthermore there was a definite movement away from the mitosis after anaphase. There was a relationship between the

Fig 4.1 Haematoxylin stained tumour culture showing a lymphocyte (L) near a mitotic tumour cell (M). Magnification x1865 oil immersion



culture conditions and mitotic attraction of lymphocytes. Mitotic attraction occurred only if coverslips were held together. This was realised only after separating of sandwiched coverslips (in order to follow and track lymphocytes on single monolayers) had been carried out for a long time with no evidence of mitotic attraction.

Used with caution in some circumstances, morphology is useful in discriminating between lymphocytes and macrophages. Unfortunately the morphology of motile cells beneath cultured epithelial cells could not be used accurately to distinguish between lymphocytes and macrophages. Cell morphology changed according to the nature of the overlying monolayer. Motile cells travelling at the cell borders of the monolayer were extruded and appeared to be following the cell border. This may be a form of contact guidance.

DISCUSSION

Elongation of cells during contact guidance is characteristic of cells moving along grooves on a substratum described by Dunn (1982). Motile cells appeared larger and fatter if they were beneath large monolayer cells, becoming small and elongated beneath small monolayer cells. In the latter case motile cells were easily within reach of cell borders, where contact guidance would occur. Dow *et al*, (1987) suggest that cells align so as to minimize distortions in their cytoskeleton. This implies that cells tend to find the flattest or least curved area of substratum.

Proliferating cultures of endothelial cells have also been demonstrated to have a strong influence on lymphocyte locomotory behaviour. Ager *et al*, (1988) report that migration of lymphocytes only occurs on live cultures,

in contrast to binding of lymphocytes to cultures, which occurs even after fixation.

The pleomorphic morphology of bizarre cultures is also a characteristic of many malignant tumours *in vivo*. It is usually reported as an indication of aggressiveness, although this may sometimes be belied by the indolent character of the growth. These results in tissue culture suggest that pleomorphic morphology occurs when the culture is not growing well or has ceased proliferating. This may also sometimes apply to tumours *in vivo*.

CONCLUSION

This study has indicated that similar proportions of both irradiated and non-irradiated larynxes have a motile lymphocyte population. Laryngeal cultures may influence lymphocyte locomotion, but only if the culture is proliferating and if the coverslips are kept together. This conclusion was derived from several findings:

1) the fact that bizarre cultures appeared not to proliferate

2) bizarre cultures usually lacked motile lymphocytes

3) bizarre cultures did not have mitoses,

4) non-bizarre cultures on coverslips held together were found to attract lymphocytes to mitotic cells in the monolayer. No attraction was observed after coverslips were separated.

This may indicate some form of chemotaxis occurred and is investigated in Chapter 7. Primary explant carcinoma culture could be a useful model for *in vitro* lymphocyte-carcinoma cell interactions. It would be necessary to ascertain if the attraction were a taxis to the mitotic cell, rather than a

chance finding due to kinesis, which sometimes appears as a taxis, but is purely random.

Lymphocytes appeared to prefer to move near cell borders suggesting that contact guidance occurred. The appearance of lymphocytes during locomotion was a poor guide to the size of the cell and was particularly unreliable as a guide to distinguishing between lymphocytes and other immunological cells.

CHAPTER 5

THE PLANE OF LYMPHOCYTE LOCOMOTION

THE PLANE OF LYMPHOCYTE LOCOMOTION

INTRODUCTION

There was evidence in my studies that lymphocytes were dislodged from the apical surfaces of cultures. There are only a few reports of lymphocytes moving on the apical surface of cultured cells and these emphasise that usually lymphocytes are cells which are particularly non-adhesive. These reports wil be described here. The aim of this chapter is to assess if the apical surface of laryngeal cultures can act as a substratum for lymphocyte locomotion.

The term 'contact inhibition' has been described by Abercrombie (1970) as the failure of a cell to continue moving in a direction that would carry it over the surface of another cell following contact. This was acknowledged as a characteristic of many cells in tissue culture and explained the formation of coherent monolayers on plastic substrata. However, Abercrombie *et al*, (1970) have also demonstrated that sarcoma cells crawl on top of the surfaces of fibroblasts. This phenomenon has been doubted by some investigators. Harris, (1982) challenged Abercrombie's findings, but found that sarcoma cells did indeed use fibroblasts as a substratum. Furthermore, he observed that some transformed 3T3 fibroblasts could occasionally spread on the surface of untransformed 3T3 cells. This was termed **'overlapping'**. Harris concluded that overlapping did occur but seemed to be the exception rather than the rule.

Studies more closely analogous to the topic of this thesis, since they observed lymphocytes on cultured monolayers, confirmed that lymphocytes preferred to migrate beneath reticular cells (Haston, 1979) and high

endothelial cells (Ager *et al*, 1988). This was termed **'underlapping'**. These studies have also observed lymphocytes to attach to upper surfaces of monolayers. Following adhesion, lymphocytes burrowed under monolayers and there migrated more rapidly than on the apical surface. Hydrated collagen lattices or gels were used for studies of cell behaviour by Elsdale and Bard (1972). Haston *et al*, (1982) proposed that lymphocytes, studied in collagen gel matrices migrated by a nonadhesive mechanism such as the extension and expansion of pseudopodia through gaps in the matrix, using the gel rather like a climbing frame. This was unique to lymphocytes, which are very poorly adhesive compared to other lymphoid and myeloid cells. Haston also found that lymphocytes migrated more rapidly in three-dimensional collagen gels than on two-dimensional collagen surfaces. Thus, collagen gel placed on top of laryngeal cultures may provide a frame into which apically-situated motile lymphocytes would migrate. Collagen gel was a good material for this application because it was transparent allowing time-lapse filming to be carried out (Haston *et al*, 1982). Furthermore, collagen gel could be used to trap motile lymphocytes and enable SEM studies to be performed without lymphocytes becoming dislodged. This method was used successfully by Haston *et al*, (1984). So it seemed that if a collagen gel were to be placed directly on the upper surface of laryngeal cultures in this study, movement in the gel could be viewed by time-lapse video microscopy, and subsequently lymphocytes could be viewed by SEM.

In addition, low and high power time-lapse video microscopy was used to investigate whether motile lymphocytes overlapped or underlapped the monolayer in the absence of collagen gel.

METHOD

Collagen Gel

Type I collagen extracted from rat tail tendons (Sigma Chemical Co) was reconstituted from lyophilised state, by adding Dulbecco's Minimal Essential Medium. The pH of the collagen was adjusted to 7.6. The collagen was allowed to set. A block of gel, measuring $2mm^3$ was cut using a scalpel and placed over a culture, which had been recorded in time-lapse and was known to have a motile population of lymphocytes. The upper coverslip was replaced over the gel and recording resumed.

Video Recording of Collagen Gel

The movement of lymphocytes into and within a disc of collagen gel situated on top of culture was attempted by recording in time-lapse. However, as the gel was transparent it was not possible to assess if motile lymphocytes were in the gel or beneath the monolayer. Therefore, after 18 h the gel was carefully removed from the culture and placed on a plastic coverslip in the same culture medium. The movement of lymphocytes which had migrated into collagen gel could then be recorded.

Scanning Electron Microscopy

The block of collagen gel was processed for SEM as described in Appendix 3.1.1. After critical-point drying, the block was cut into six smaller specimens and each specimen orientated to display the top surface or the lower surface. Specimens were mounted on aluminium stubs using

adhesive tape (Polaron Ltd), sputter-coated with gold-palladium and viewed with a Jeol SEM-500 scanning electron microscope.

High Power Video Time-Lapse Microscopy

Cultures were grown on glass coverslips in order to improve resolution at objective power x40. Video recording was carried out in time-lapse at 10 second intervals.

RESULTS

Collagen Gel Study

The only cells detected on collagen gel after removal from culture and processing for scanning electron microscopy, were red blood cells (Fig 5.1) and two unidentified cells, which may have been lymphocytes (Figs 5.2 & 5.3). No other cells with a typical lymphocytic appearance were found adherent on collagen gels, after scanning electron microscopy was carried out. Furthermore, direct visual assay using time-lapse video microscopy failed to show any motile lymphocytes in collagen gel, following removal of the gel from the culture.

High Power Video Time-Lapse Microscopy

Normally, lymphocytes were seen to be phase-dark, have a hand-mirror morphology and move with a leading lamella and trailing uropod when motile under the culture. Lymphocytes present on top of cultures were found to be phase-bright, spherical and locomotion could not be distinguished from Brownian motion. For this reason only phase-dark lymphocytes have been

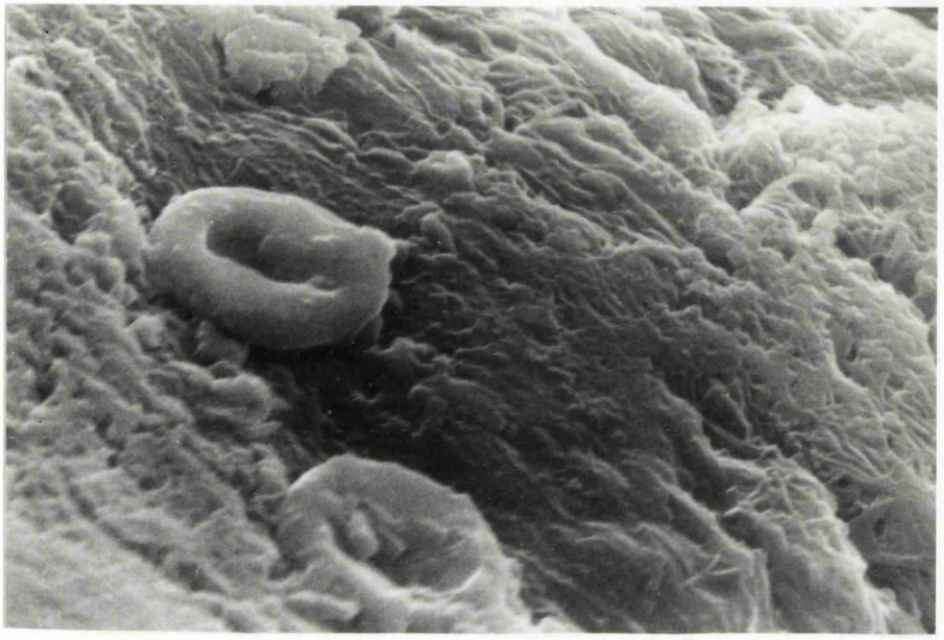
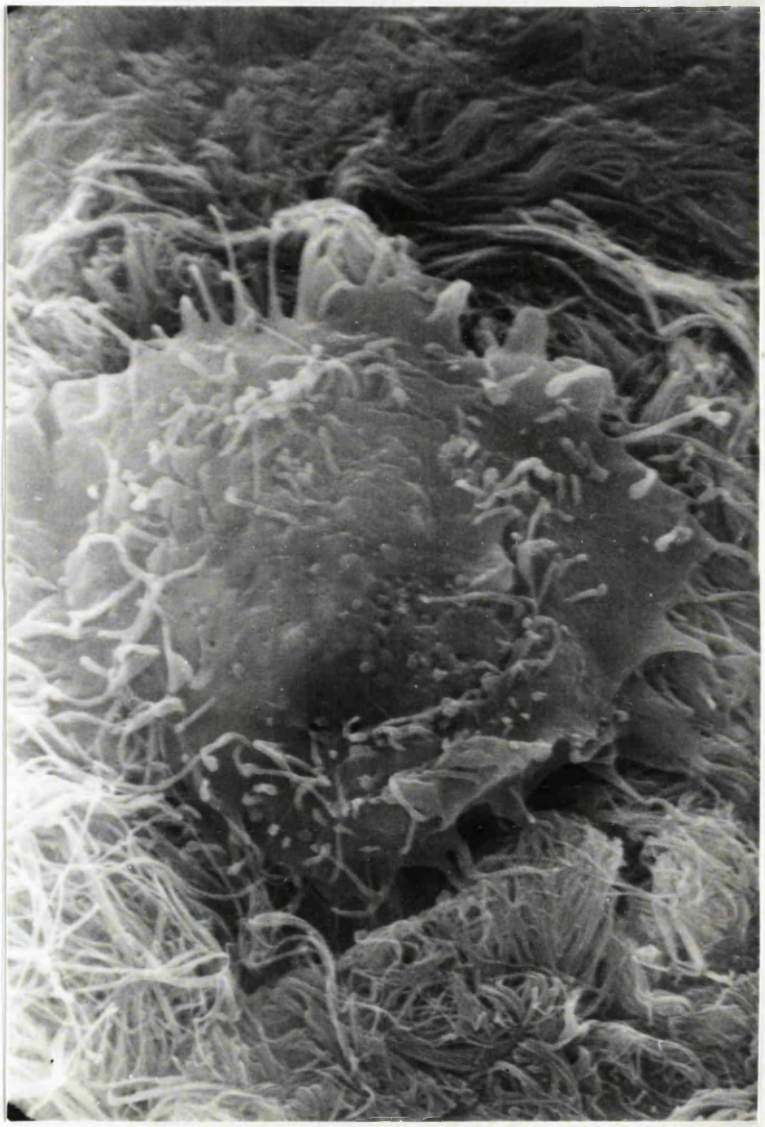


Fig 5.1 Scanning electron micrograph of red blood cells (derived from non-neoplastic culture) on collagen gel. Magnification x5797

Fig 5.2 Scanning electron micrograph of an unidentified cell (possibly a lymphocyte) on collagen gel. Magnification x4581



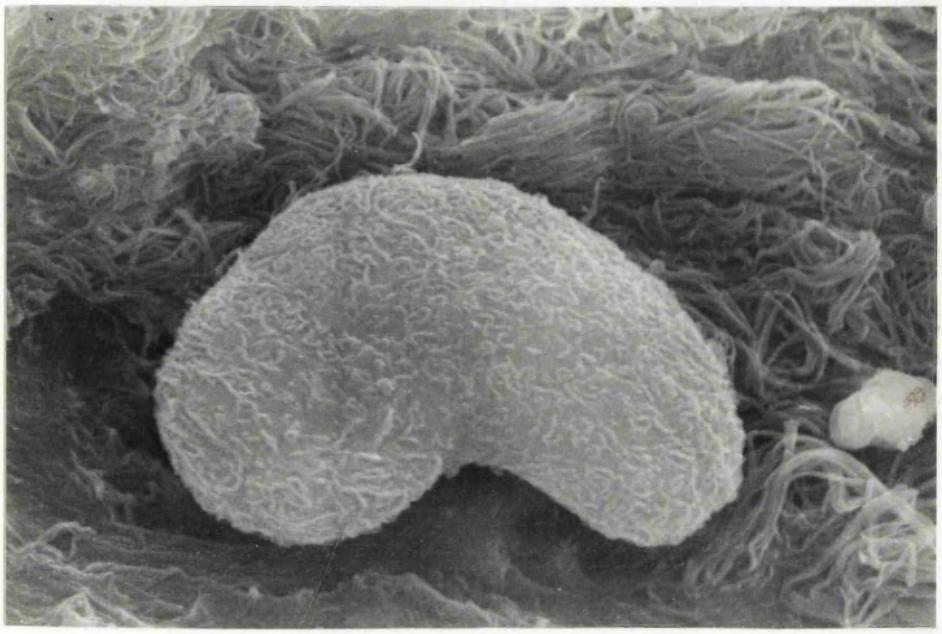


Fig 5.3 Scanning electron micrograph of a second unidentified cell (possibly a lymphocyte) on collagen gel. Magnification x4647

investigated throughout this study and had been expected to reside beneath the culture. However, by using a high power objective, it was found that some phase-dark lymphocytes are capable of a characteristic lymphoid locomotion on the apical surface of laryngeal cultures. There are several sequences of video tape, which show that there are undoubtedly some motile and phase-dark lymphocytes on the apical surface of laryngeal cells.

The video at time code 00:13:27 shows a sequence recorded at low-power with a x10 objective. This was a normal true cord culture on which there was a single motile lymphocyte. The lymphocyte appeared to cause a loose piece of debris to shift slightly on the two occasions that it was underlapped by the lymphocyte. To prove that the debris was on the upper surface of the culture, the debris was dislodged by creating strong convection currents in the medium with a Pasteur pipette.

The following sequences were recorded at high power using a x40 objective. The video at time code 00:07:53 shows a motile lymphocyte emerging from a basolateral position out of a gap or rupture in monolayer of true cord culture. This showed that the lymphocyte became deformed as it squeezed its leading lamella and body through the narrow opening. As it emerged, the leading lamella became phase-bright and appeared to lose contact with the culture for a brief period. The body of the lymphocyte constricted as it migrated out of the gap in the culture and finally pulled its uropod through. After the whole lymphocyte had crawled out onto the apical surface, the lymphocyte was seen to become phase-dark as it spread and adhered to the monolayer. It remained phase-dark and proceeded to migrate on the apical surface apparently without difficulty, in the same fashion as seen when migrating under the culture. This sequence shows that if a lymphocyte has spread on either the apical or basolateral surface it

appeared phase-dark. Also this showed that a lymphocyte in the basolateral location has no option but to spread and therefore appeared flattened. However a lymphocyte on the apical surface was seen to behave differently and could appear either phase-bright or phase-dark. The leading lamella became phase-bright which indicated that it was not adherent and there was no locomotion seen, until the entire lymphocyte was pulled out from beneath the culture and could spread on the substratum.

Phase-dark appearance is demonstrated to be a feature of lymphocytes when underlapping **and** overlapping culture. One motile lymphocyte on tumour culture was seen to attempt unsuccessfully to burrow beneath a tumour cell (see video at time code 00:11:30). A distinct flattening occurred as the lymphocyte migrated and underlapped a tumour cell. Also the outline of the lymphocyte was less clearly defined, than when it withdrew and was moving on the apical surface.

Further evidence of the flattening that occurs and helps the lymphocyte gain access to basolateral areas is shown in the video at time code 00:03:35. A lymphocyte becomes more flattening to crawl beneath the edges of epithelial cells.

DISCUSSION

Collagen gel did not show lymphocyte migration on the apical surface. This experiment was carried out only on cultures which were known to have a large number of motile lymphocytes which would be expected to colonise the gel more readily than sparsely populated cultures. Perhaps all these lymphocytes were situated basolaterally. More success was achieved from a small number of video sequences. It was confirmed that lymphocytes

appeared phase-dark when moving on the apical and basolateral positions of tumour and normal cultures. This occasional sighting of apically situated motile lymphocytes would tend to enhance Harris' finding that lymphocytes usually move beneath the culture, although overlapping does occasionally occur.

The upper cell surface of epithelial cells in culture was shown to be a particularly non-adhesive substratum by DiPasquale *et al*, (1974). Their work concerned fibroblasts adhering to epithelial cells, but their assumptions are applicable to this chapter. They found some adhesion to the upper cell surface, following initial adhesion at the cell margin. This was explained as an effect of the centripetal movement of adhesion sites established first at the cell margin. More appropriately, Chang's group (1979) found that lymphocytes crawl along the margins of fibroblasts. This is probably due to the same type of adhesion zones at the margin of fibroblasts as described for epithelial cells by DiPasquale's group. It is a possibility that lymphocytes crawling on epithelial cells are taking advantage of similar adhesion sites.

CHAPTER 6

THE IDENTIFICATION OF MOTILE LYMPHOCYTES

THE IDENTIFICATION OF MOTILE LYMPHOCYTES

I INTRODUCTION TO TRACKING

Tracking of Motile Lymphocytes

Upon immunological stimulation, lymphocytes from all sources become highly motile. Chang *et al*, (1979) used live cell microscopy, to observe lymphocytes crawling on fibroblast cultures and estimated that crawling could be as rapid as 20 μm/min. They concluded that movement was easily detectable without time-lapse apparatus. However, a need for time-lapse recording of cell movement was recognised where cell interactions were more complex. Furthermore, time-lapse microscopy was useful to locate and investigate precise motile cells and distinguish them from non-motile forms. This was termed 'tracking'. Following detection of motility by time-lapse filming, tracked lymphocytes were investigated by TEM and SEM by Haston *et al*, (1984) to show the constriction ring in motile lymphocytes, immunofluorescence by Wilkinson, (1985) to identify the phenotype of motile lymphocytes, or immuno-electron microscopy by Balfour *et al*, (1989) to investigate relationships between antigen-presenting veiled cells.

In the study described in this thesis, video time-lapse apparatus was found to be a useful tool for tracking motile lymphocytes. Attempts were made to study tracked lymphocytes by many of the methods cited in the preceding review of the literature.

METHOD

Locomotion of lymphocytes on laryngeal cultures was identified by re-playing time-lapse recording at normal tape speed, so that real time events were speeded up. Details concerning orientation of motile lymphocyte with respect to culture, explant and coverslip substratum were quickly sketched and photographed. The culture was fixed as soon as possible.

Fixative

The choice of fixative depended on the investigation to be carried out. Details of fixatives are included in Appendix 1 and 3.

Investigations Carried Out on Tracked Lymphocytes

Leishman Stain

Cultures were stained with Leishman stain to reveal lymphocyte morphology.

Scanning Electron Microscopy

Scanning electron microscopy was carried out to attempt to describe the appearance of motile lymphocytes. Cultures were processed as described in Appendix 3.1.1. An arrow was scratched on the bare coverslip close to the tracked lymphocyte.

Immunological Staining

Indirect immunoperoxidase anti-peroxidase or alkaline phosphatase anti-alkaline phosphatase methods were used in conjunction with lymphocyte phenotype subset monoclonal antibody markers. Some fluorescein-conjugated monoclonal antibodies were also used. The latter required that glass coverslips were used and that cultures were fixed in 1% paraformaldehyde in phosphate buffered saline at pH 7.4. These methods are described in greater detail in Appendix 3.2-3.6. Cultures were counterstained with haematoxylin.

SUMMARY

Results showed that SEM was unsuitable for tracking lymphocytes, but that immunocytochemical techniques and Leishman staining were good methods. The absence of some lymphocytes from cultures stained by the latter techniques was confirmed by counterstaining. This suggested that lymphocytes were dislodged from the monolayer and may explain why SEM was unsuitable, since this technique required extra processing. An investigation of the position of lymphocytes with respect to the culture surface was therefore carried out as described in Chapter 5 .

2 INTRODUCTION TO STAINING

Immunocytochemical Staining of Lymphocytes

Time-lapse recording showed that cells motile on cultures were derived from the explant tissue. Staining of motile cells with haematoxylin or Leishman indicated a lymphocytic appearance. The study described in this chapter commences with an attempt to identify phenotypes of motile lymphocytes. The monoclonal antibodies UCHT1, UCHT4, Leu3a and 4KB128, which label Pan T cells, CD8+ T cells and CD4+ T cells and B cells, were employed.

This study also included markers for other motile immunological cells. Zeromski *et al*, (1986) had found that laryngeal tumour sections were infiltrated by macrophages and natural killer cells (NK), in addition to T lymphocytes and occasionally B lymphocytes. The ability of macrophages to migrate has been studied by Wilkinson, (1982). Cultures and sections were stained with macrophage markers (Leu3M and P150.95, respectively). Similarly markers NKH1 and HNK1 were included. These labelled NK cells and large granular lymphocytes (LGL). NK and LGL motility has been demonstrated by Natuk *et al*, (1987).

A subset of lymphocytes, known as intraepithelial lymphocytes (IEL) has recently been found in gut mucosa. IEL were shown to be motile *in vitro* but non motile *in vivo* by Baca *et al*, (1987). Cerf-Bensussan *et al*, (1987) described a monoclonal antibody (HML-1) defining a novel membrane molecule present on human IEL. This has also been detected on IEL located between carcinomatous cells of the gut by Jarry *et al*, (1988). It was not known if HML-1+ lymphocytes infiltrate other tissues. As laryngeal

squamous cell carcinoma is derived from endodermal epithelium, HML-1 was included in the panel of monoclonal antibody markers.

Other monoclonal antibody cell markers were included because the cells have been associated with motility. These included anti-IL-2 for activated T cells, null cell marker (CD7), class II antigen (HLA-DR) marker and Ki67 which labels proliferation antigen and therefore would identify lymphoblasts.

A small study of infiltrates in sections of laryngeal tissue was carried out. This employed all monoclonal antibodies previously included in this study to identify motile cells.

METHODS

Preparation of Control Sections

Serial cryostat sections of 10 μm thickness were cut from laryngeal tumour and true cord specimens. Sections were mounted on poly-L-lysine coated slides. Sections were air-dried for 1 hour at room temperature and fixed in cold acetone for 30 min. Sections were stored at -20°C.

Preparation of Cultures

Cultures and (where possible) explant tissue were left adhered to coverslips and fixed in acetone for 5 min and air-dried. Coverslips were glued to glass slides so that culture faced upwards. Cultures were stored at -20°C in air-tight containers.

Immunocytochemical Tests

PAP and APAAP Techniques

Frozen cultures and sections were defrosted at ambient temperature and incubated with monoclonal antibodies to the surface antigens on lymphocytes, macrophages and natural killer cells by the indirect immunoperoxidase anti-peroxidase (PAP) method or alkaline phosphatase anti-alkaline phosphatase (APAAP) method, as described in Appendix 3.2-3.4. The viability of monoclonal antibodies was tested during each staining session using laryngeal tumour sections. Alternative positive controls were required to test for antigens not commonly found on laryngeal tumour infiltrates. Sections of tonsil and small intestine were used to test markers of B cells and HML-1 cells, respectively. The specificities of monoclonal antibodies are listed in Table 6.1. Single stain procedure or sequential double stain procedures were employed. The latter are described in Appendix 3.5.1 & 3.5.2. Positive cell infiltrates were enumerated accurately as a percentage and approximately using a +/- scale. Attempts were made to select fields representative of intense as well as scattered infiltrates. Specimens stained with the chromagen fast red or fast blue were mounted in an aqueous mountant such as Apathy's mountant (BDH Ltd.). Other specimens could be adequately mounted in RAL mount (Raymond A. Lamb, London).

Immunofluorescence Technique

Cultures were incubated with a small panel of fluorescein-conjugated monoclonal antibodies (UCHT1, UCHT4, Leu3a and TCRδ-1), as described in Appendix 3.6. Specimens were mounted in glycerol. To prevent glycerol drying up, coverslip edges were sealed onto glass slides using varnish. Specimens were examined with a Leitz fluorescent and phase-contrast microscope.

TABLE 6.1

SPECIFICITIES OF MONOCLONAL ANTIBODIES USED IN THE IDENTIFICATION OF MOTILE CELLS

MAB	CLUSTER DIFFERENTIATION	SPECIFICITY
UCHT1	CD3	Pan T cells
UCHT4	CD8	cytotoxic/suppressor T cell
Leu3a	CD4	helper T cells and some macrophages
	CD7	null T cells
Leu3M		macrophages
P150.95		macrophages
4KB128	CD22	B cells
αIL2		activated T cells, some macrophages
TCRδ-1	CD3+/CD4-/CD8-	double negative CD3 T cells
HML1		IEL
HNK1		NK cells and large granular lymphocytes (LGL)
NKH1		NK cells
Ki67		proliferation antigen
IB5		HLA-DR antigen

Sections Used as Controls for Monoclonal Antibodies

Since the number of cultured preparations available from each larynx was never more than five, only one or two markers could be duplicated per staining session. The number of control tests available was only one tumour section for each marker plus one tumour section to be used as a negative control, in which tris buffered saline solution (TBS) was used in place of antibody.

Sections Used in a Separate Study of Laryngeal Infiltrates

In a parallel study, laryngeal infiltrates in sections were stained with the complete panel of antibodies applied to cultures. Sections studied were from two tumour cases (cases 1 & 2) and one normal true cord (case 1). Two further tumours (cases 3 & 4) were tested for CD4, CD8 and CD3 infiltration. These were initially assessed with a x20 objective and taking score using a scoring system of 0,+/-,+ or ++. A further more accurate quantitative method was achieved by counting positive cells in a x40 field, as a percentage of the total number of cells in the same field. The arithmetic mean of ten fields varying from low to high intensity infiltration was calculated.

Tracking of Motile Cells

Preliminary attempts to track motile cells were carried out prior to counterstaining and mounting, to assess the intensity of the fast red chromagen used in the APAAP technique. This was necessary because control cells in sections were not analogous to cells in culture and stained with different intensity. If stain was found to be weak, minimal counterstain

was applied. Counterstaining with haematoxylin was necessary to distinguish monolayer features. This enabled more accurate tracking of cells in relation to each monolayer arrangement. Cultures stained by immunofluorescence were not counterstained. Attempts to track motile cells in these cultures was carried out using phase-contrast and fluorescence optics. Photomicrographs and diagrammatic representations of the monolayer in the vicinity of the motile cell were compared to time-lapse sequences and the stained culture.

RESULTS

Phenotypes of Infiltrates in Laryngeal Sections

Haematoxylin and eosin stains revealed foci of lymphocytes in tumour parenchyma (Fig 6.1) and around the tumour (Fig 6.2). Results of attempts to phenotype the leucocytic infiltrates in four laryngeal tumours are tabulated in Tables 6.2-6.5. In most cases, tumours were predominantly infiltrated by macrophages and helper/inducer T cells in the tumour parenchyma. In peri-tumour regions, helper T cells, macrophages and suppressor/cytotoxic T cells and Pan T cells were detected. In some tumour parenchyma there was also a scanty distribution of suppressor/cytotoxic T cells, null cells (CD7), TCRδ-1 and B cells. No natural killer cells or HML-1 cells were found. Predominantly macrophages were found in the normal epithelium, with some helper/inducer T cells, suppressor/cytotoxic T cells and CD7 cells. No TCRδ-1 cells, B cells or HML-1 cells were found in normal epithelium. A study of infiltrates in normal laryngeal connective tissue was also carried out. Helper/inducer T cells and macrophages were found to be most predominant. CD7 cells, B cells and TCRδ-1 cells were found in scanty distribution.

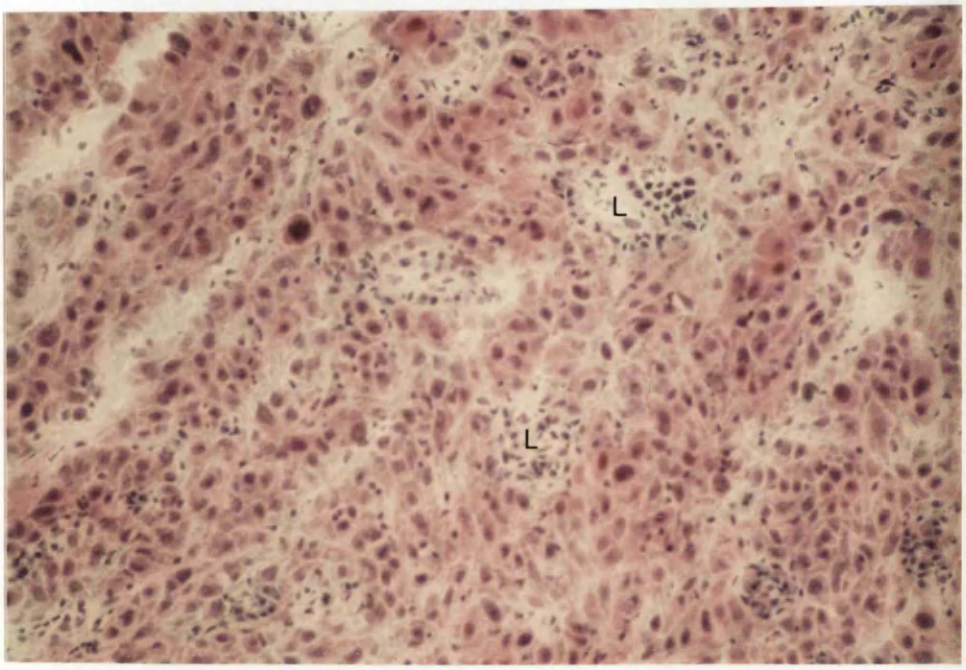


Fig 6.1 Haematoxylin and eosin stained tumour section. Foci of lymphocytes in the parenchyma (L). Magnification x360

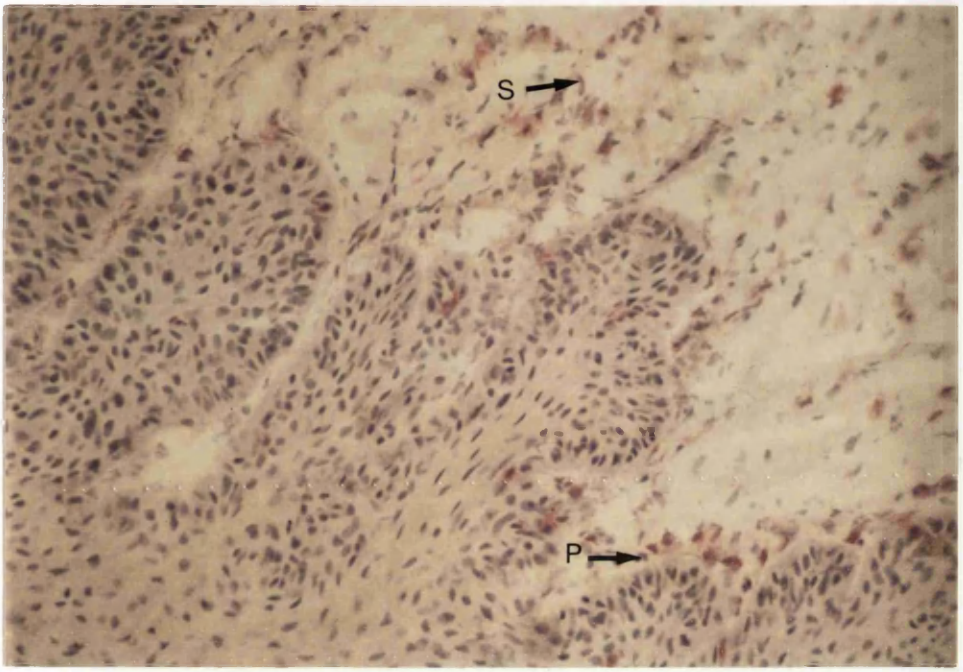


Fig 6.2 Haematoxylin and eosin stained tumour section. Lymphocytes accumulate at peri-tumour region (P) and in the stroma (S). Magnification x360

TABLE 6.2

COMPARATIVE ASSESSMENT BY +/- SCALE OF INFILTRATES IN TUMOUR, PERI-TUMOUR, NORMAL EPITHELIUM & CONNECTIVE TISSUE

CASE 1

MONOCLONAL	EPITHELIUM				TUMOUR				CT				PERI-TUMOUR			
ANTIBODY	0	+/-	+	++	0	+/-	+	++	0	+/-	+	++	0	+/-	+	++
UCHT1			+				+				+				+	
UCHT4		+					+				+				+	
Leu3a		+						+				+				+
HML-1	+						+				+				+	
CD7		+					+				+				+	
NKH1	+						+				+				+	
P150.95			+					+				+				+
4KB128	+						+				+				+	
TCRδ-1	+						+				+				+	

CT = connective tissue

For specificities of monoclonal antibodies see Table 6.1

TABLE 6.3

A +/- SCALE OF INFILTRATES IN CASE 2 TUMOUR

CASE 2

MONOCLONAL ANTIBODY	TUMOUR 0	+/-	+	++
UCHT1		+		
UCHT4		+		
Leu3a			+	
P150.95			+	
HML1	+			
CD7		+		
4KB128		+		
NKH1	+			
TCRδ-1		+		

TABLE 6.4 PERCENTAGE OF POSITIVELY STAINED INFILTRATES IN THREE LARYNGEAL CARCINOMAS.

MONOCLONAL ANTIBODY	POSITIVE CELLS PER 100 TUMOUR CELLS		
	CASE 2	CASE 3	CASE 4
UCHT1	1.47	NT	3
UCHT4	1.6	4.9	NT
Leu3a	3.7	18	5.5
P150.95	2.2	NT	NT
HML1	0	NT	NT
CD7	1.5	NT	NT
4KB128	1.95	0	NT
NKH1	0	NT	NT
TCRδ-1	0.5	NT	0.2

NT = not tested

TABLE 6.5 PERCENTAGE OF INFILTRATES WITHIN TUMOUR AND PERITUMOUR IN TWO LARYNGEAL CARCINOMAS (CASES 3 & 4).

	CASE 3		CASE 4	
MONOCLONAL ANTIBODY	TUMOUR	PERITUMOUR	TUMOUR	PERITUMOUR
UCHT1	NT	NT	3	53
UCHT4	4.9	17.4	NT	NT
Leu3a	18	12	5.5	22.6

Some Lymphocytes Identified in Explants

During the course of staining cultures, some explant tissue still remained attached to several cultures. HML-1 and cytotoxic/suppressor T cells were identified with red blood cells in blood vessels, as shown in Figures 6.3 & 6.4, 6.5, indicating that motile lymphocytes may be derived from the vasculature. This staining was not carried out routinely, because the explant was usually lost from the coverslip.

One culture and explant stained with IB5 a monoclonal antibody marker for HLA-DR antigen showed that the culture and motile cell were negative, but the vasculature was positive (Fig 6.6).

Identity of Motile Cells

In cultures of tumour, only T cells (Fig 6.7) and cytotoxic/suppressor T cells (Fig 6.8) were identified to be motile (see video at time codes 00:14:09 and 00:15:00, respectively). Cultures of true cord were found to have motile Pan T cells (Fig 6.9), cytotoxic/suppressor T cells, helper/inducer T cells (Fig 6.10), CD7 cells and HML-1 cells (see Figure 6.11 and video at time code 00:15:15) . No motile cells were positive for antibodies specifically marking B cells, macrophages or natural killer cells in either tumour or control culture.

The panel of monoclonal antibodies was reviewed in the light of recent literature pertaining to new monoclonal antibodies, which stained tumour infiltrating lymphocytes (HML-1, as described) and other infiltrating immunological cells (TCRδ-1). The panel included every possible T cell marker and markers for lymphoid and myeloid motile cells or cells of

unknown motility. TCRδ-1 was recently identified as a polypeptide expressed on a population of T cells, which are unusual in that they express high levels of surface antigen CD3, but are CD4-/CD8-. These cells also express a novel heterodimer γ-δ T cell receptor (Brenner *et al*, 1986).

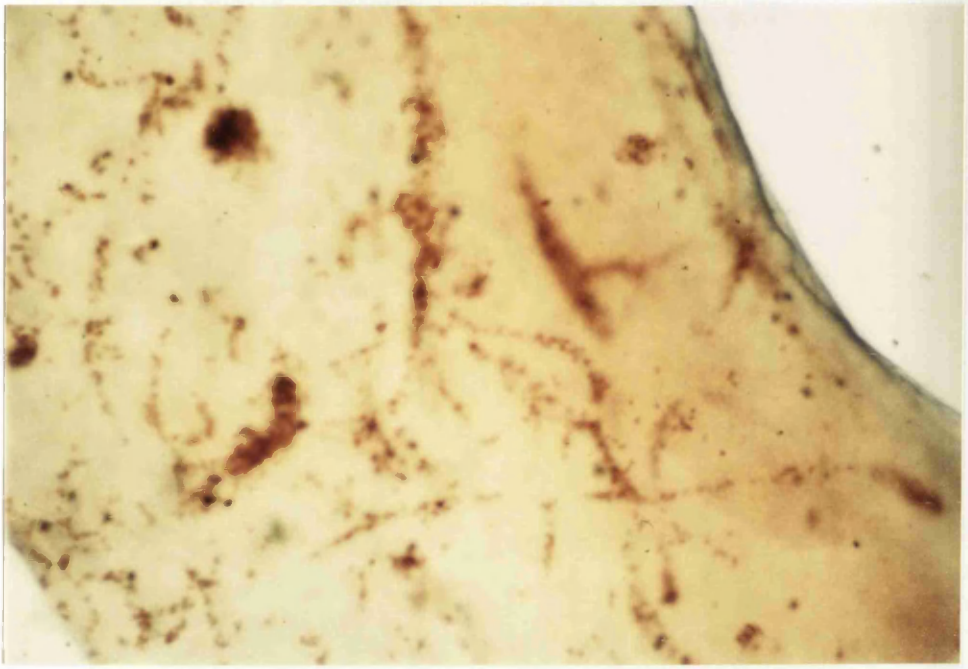


Fig 6.3 This explant of normal true cord has stained positively with HML-1 monoclonal antibody in the vasculature by the indirect PAP method, using diaminobenzidine as chromagen. Magnification x360

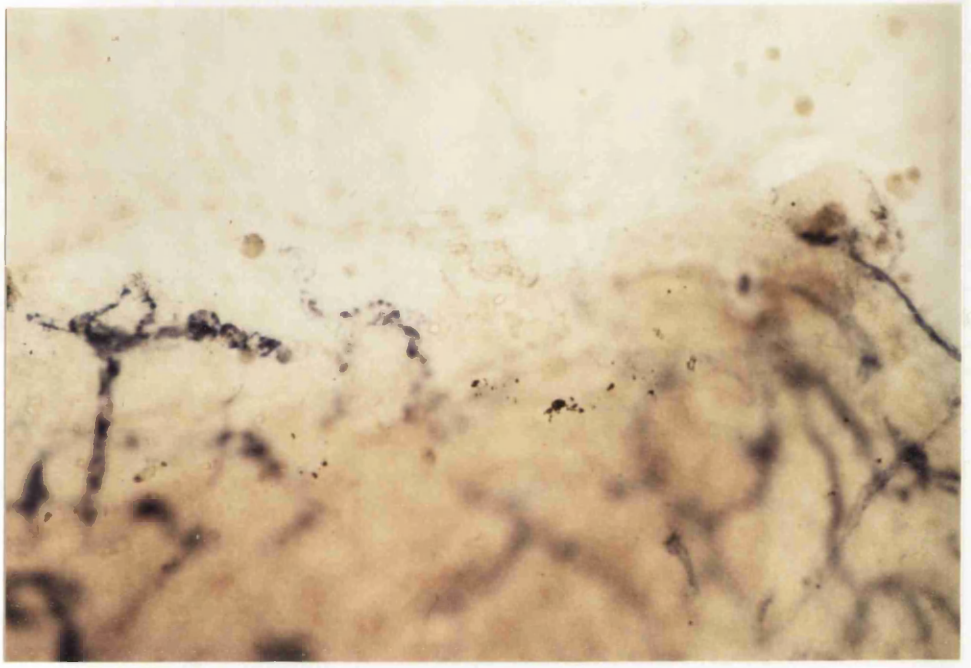


Fig 6.4 Explant of normal true cord stained with UCHT4 monoclonal antibody by APAAP technique using fast blue chromagen. Magnification x900

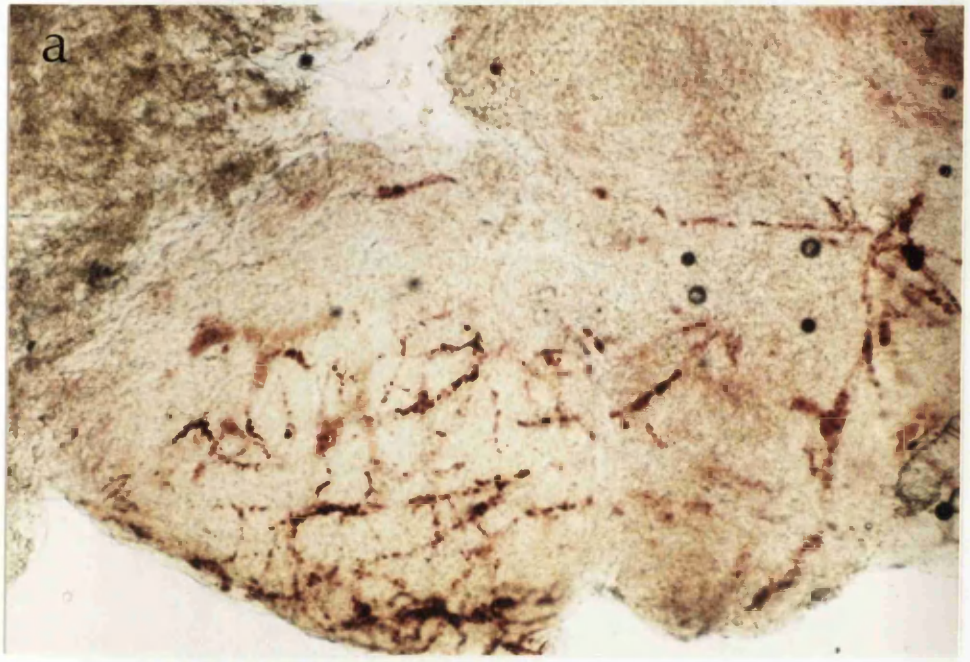


Fig 6.5 a) & b) Explant of normal true cord stained with UCHT4 monoclonal antibody by APAAP technique using fast red chromagen. Magnification a) x360 & b) x3600 oil immersion.

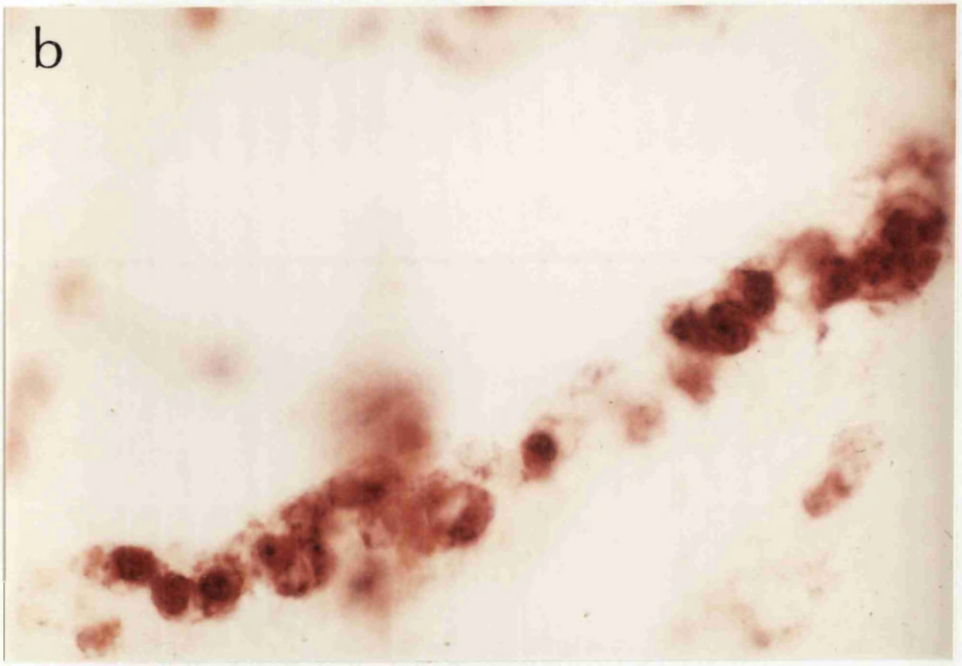
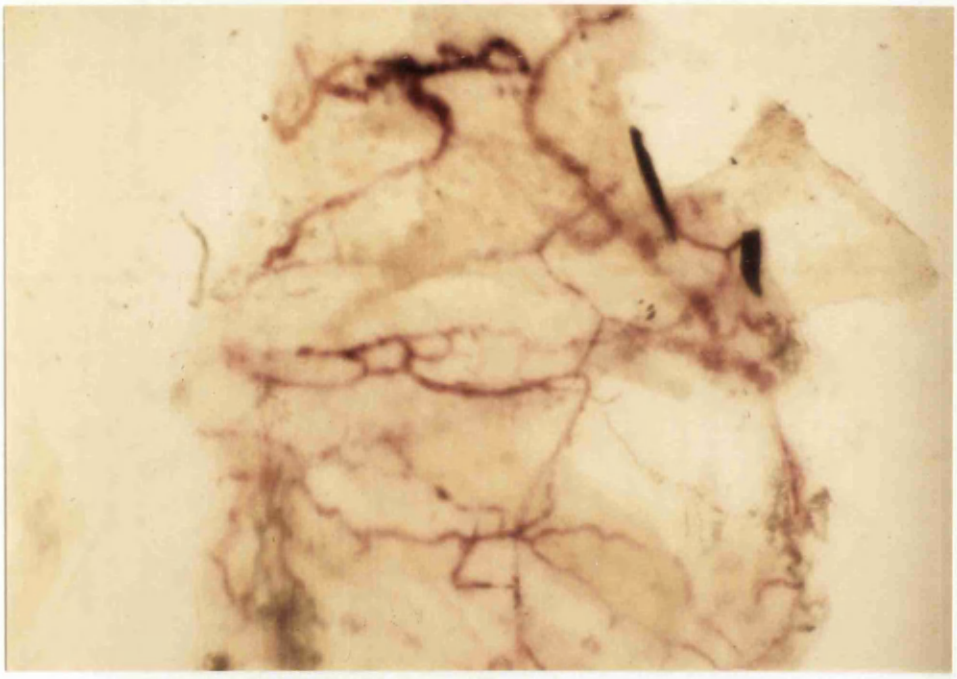


Fig 6.6 Explant of tumour stained with IB5 monoclonal antibody which identifies HLA-DR antigen in the vasculature. APAAP technique using fast red chromagen. Magnification x360



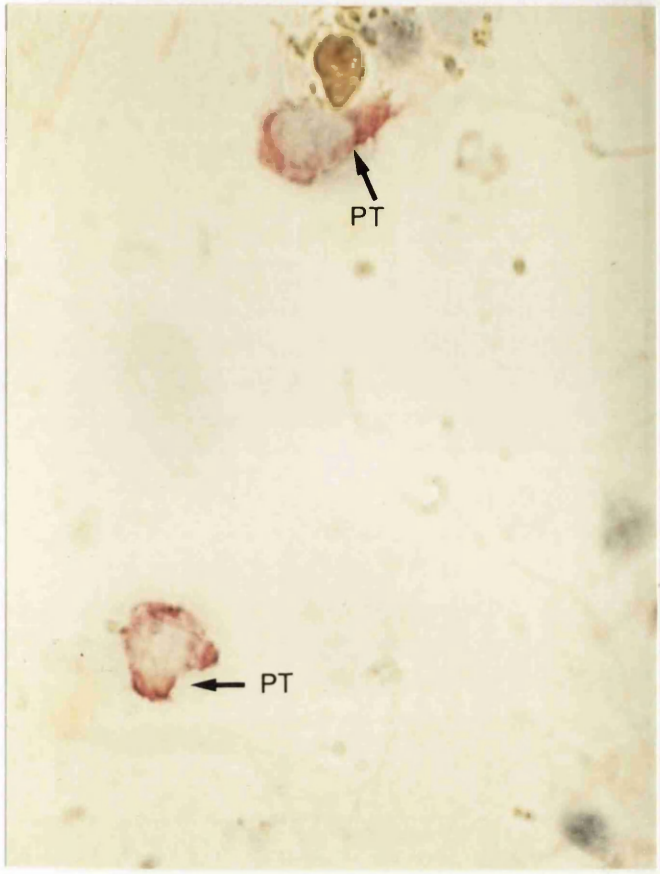
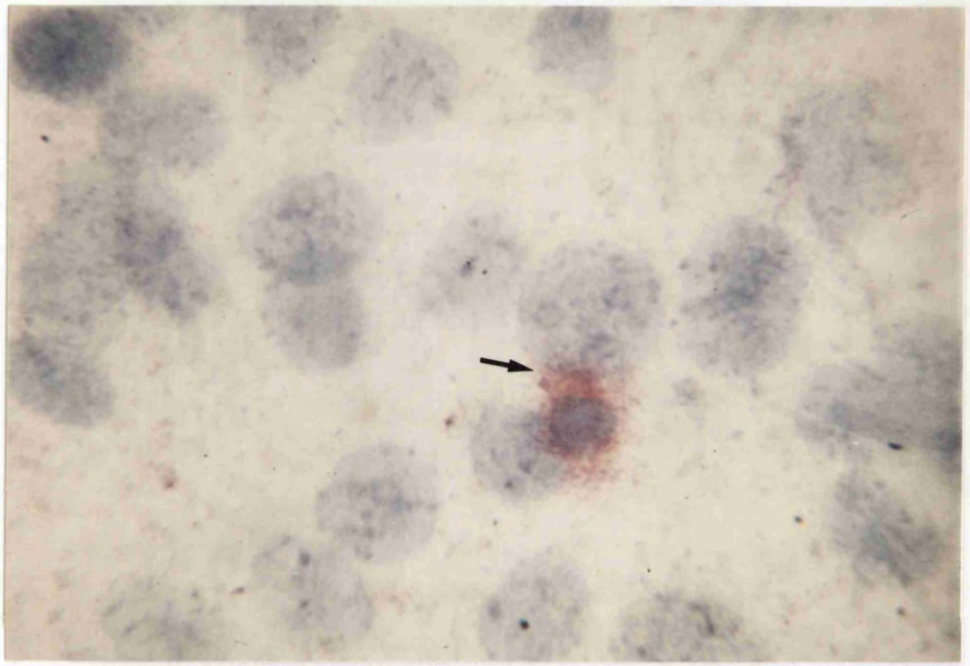
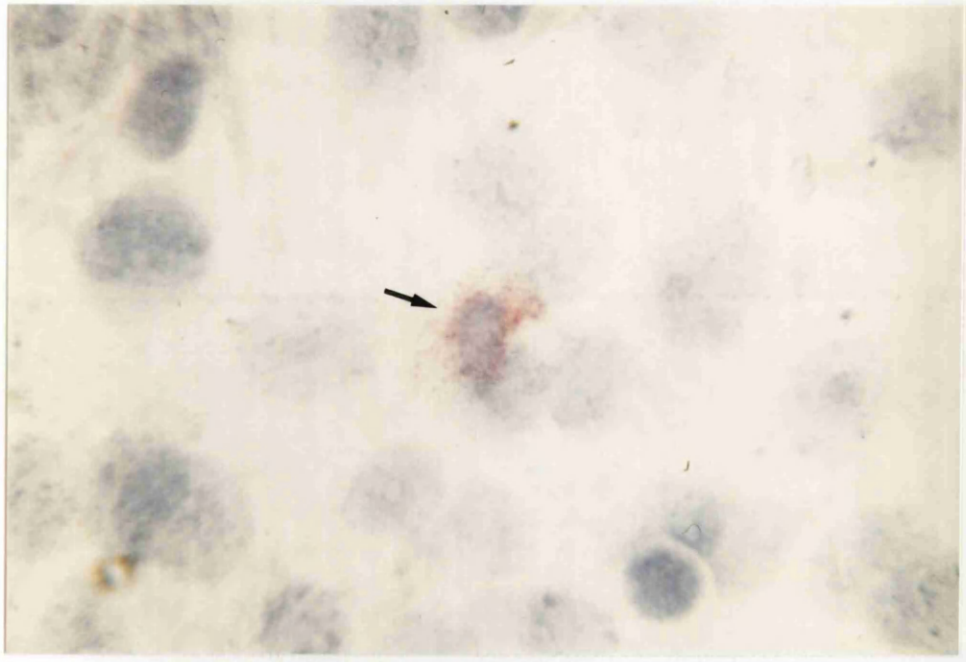


Fig 6.7 Tumour culture stained with monoclonal antibody UCHT1 by APAAP technique and fast red chromagen; counterstained with haematoxylin. Positive cells are Pan T lymphocytes (PT) which were motile. Magnification x3600 oil immersion.

Fig 6.8 a) & b) Tumour cultures stained with monoclonal antibody UCHT4 by APAAP technique using fast red chromagen; counterstained with haematoxylin. These suppressor/cytotoxic T lymphocytes were motile cells. Magnification x3629 oil immersion.





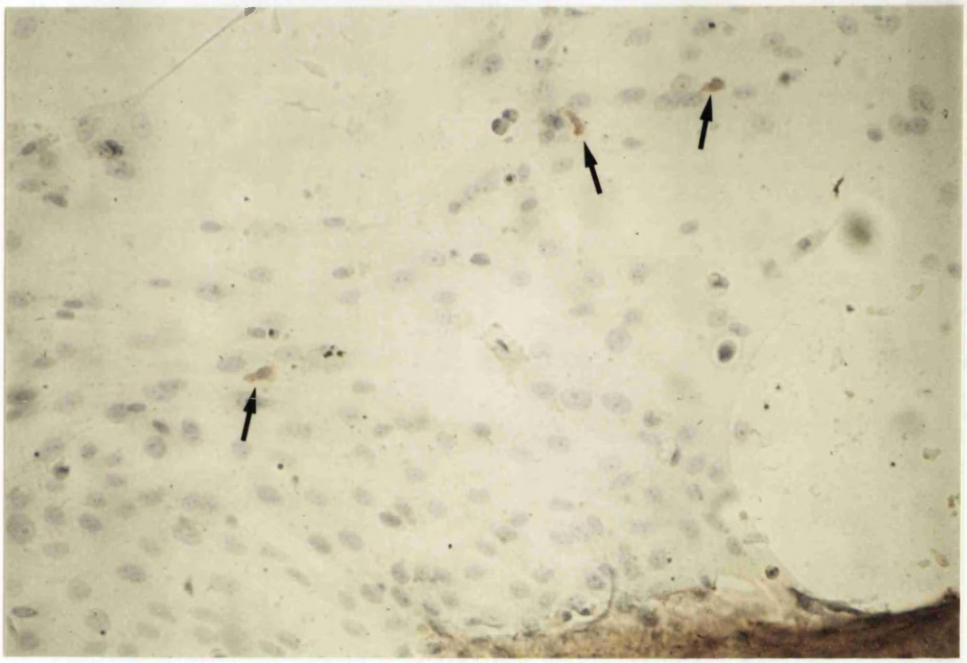


Fig 6.9 True cord culture stained with UCHT1 monoclonal antibody by indirect PAP technique and diaminobenzidine chromagen. Counterstained with haematoxylin. Positive cells, which are brown, are motile Pan T lymphocytes. Magnification x576

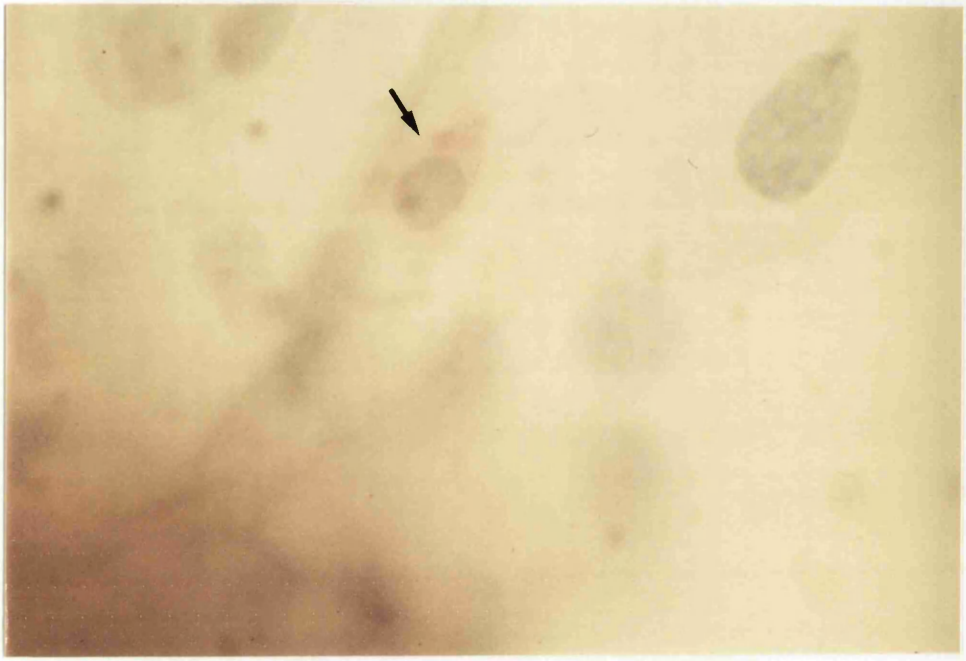


Fig 6.10 True cord culture stained with Leu3a monoclonal antibody by APAAP technique using fast red chromagen. Counterstained with haematoxylin. This positive cell, which was motile has the appearance of a lymphocyte and is therefore a helper/inducer T lymphocyte. Magnification x3600 oil immersion.

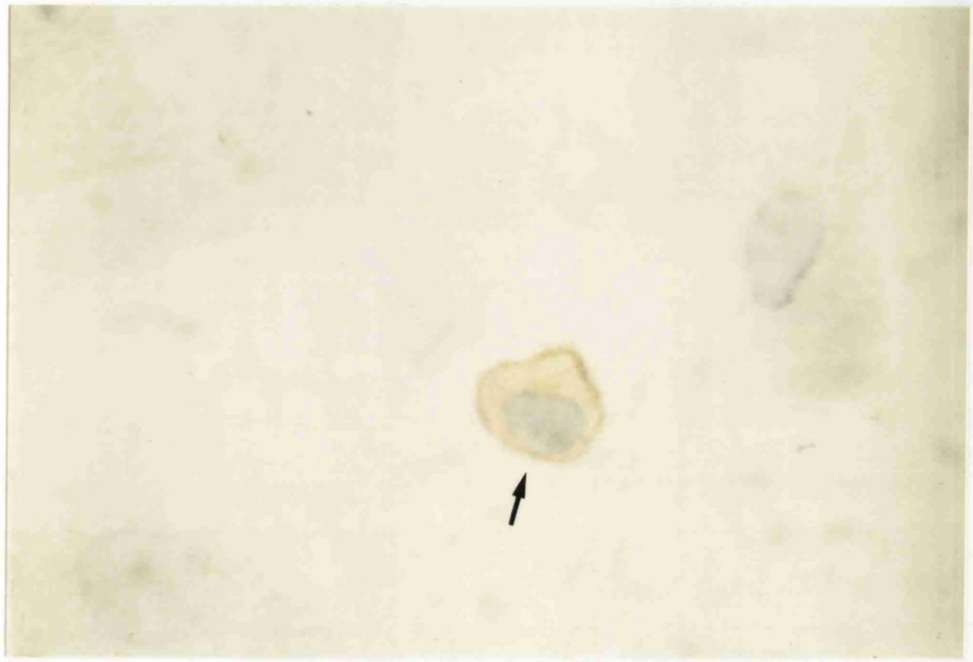


Fig 6.11 True cord culture stained with HML-1 monoclonal antibody by indirect PAP technique and diaminobenzidine chromagen. Counterstained with haematoxylin. Positive cell (stained brown) is a motile intraepithelial cell. Magnification x3686 oil immersion.

In case the motile cells which did not label with sequential stains of UCHT4 and UCHT8, were TCRδ-1 cells this antibody was included. No positive cells were identified.

Table 6.6 and 6.7 show the number of motile cells which stained positively or negatively. A summary of results of cultures after staining is in Appendix 2 Table 9. There were many cells which did not stain, despite carrying out double staining procedures in some cases, in which two markers were applied in sequential steps to each culture. Initially this suggested that subset markers did not represent a broad enough range. However, after using many other markers listed in Table 6.6, there were still many unidentified motile cells. Reasons for this were investigated. Since the panel of markers was broad it seemed highly unlikely that inadequate tests were carried out. A further example suggested that there were areas of the monolayer which required additional permeabilisation before monoclonal antibody could penetrate to the basolateral cell surface. Figure 6.12 shows a culture, which had four motile cells: three of which stained Pan T cell positive. One cell was negative. Upon examination there was no explanation for this. All cells had been motile on the same surface of the culture i.e. the basolateral surface. As the marker for Pan T cells, UCHT1, had the broadest specificity of all the monoclonal antibodies used and most of the motile cells previously identified were Pan T cell positive, some factor associated with the monolayer was thought to be responsible for this difficulty.

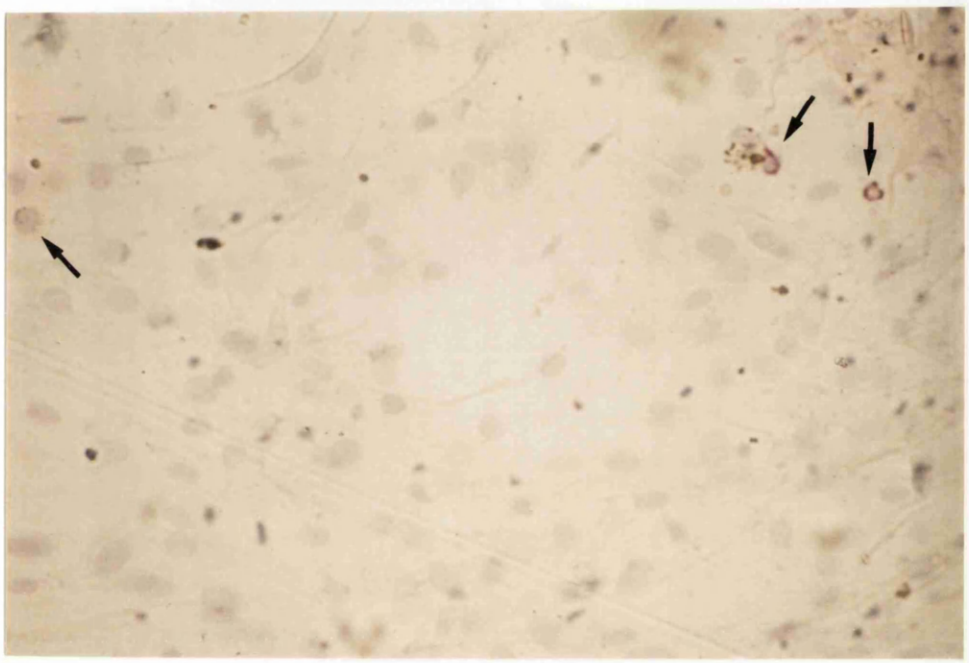


Fig 6.12 Tumour culture stained with UCHT1 monoclonal antibody by APAAP technique and fast red chromagen; counterstained with haematoxylin. Three out of four motile cells were identified as Pan T lymphocytes. Magnification x360

TABLE 6.6 POSITIVELY AND NEGATIVELY STAINED MOTILE CELLS IN TRUE CORD OR OTHER CONTROL CULTURE

MAB	NUMBER OF CULTURES	POSITIVE CELLS	NEGATIVE CELLS
UCHT1	5	4	6
UCHT4	7	9	25
Leu3a	6	3	30
CD7	3	1	5
Leu3M	1	0	1
4KB128	1	0	4
αIL2	1	0	1
TCRδ-1	1	0	4
HML1	12	2	14
HNK1/NKH1	3	0	5
Ki67	3	0	4
TOTAL	43	19	99

TABLE 6.7

POSITIVELY AND NEGATIVELY STAINED MOTILE CELLS IN TUMOUR CULTURE

MAB	NUMBER OF CULTURES	POSITIVE CELLS	NEGATIVE CELLS
UCHT1	5	9	25
UCHT4	5	2	8
Leu3a	6	0	43
CD7	NT		
Leu3M	1	0	1
4KB128	1	0	2
αIL2	NT		
TCRδ-1	2	0	14
HML1	1	0	4
HNK1/NKH1	3	0	19
Ki67	NT		
TOTAL	24	11	116

DISCUSSION

Study of Infiltrating Cells in Sectioned Laryngeal Tissue.

This study was not intended to be a comprehensive investigation of laryngeal tumour infiltrates, since this has been carried out by several groups (see Chapter 2 Literature Review and Introduction to this chapter). but was intended as a control for infiltrates stained in explants. The study demonstrated that antibodies also marked infiltrating cells in sections and indicated which cells might be expected to be present on cultures. Indeed certain differences and similarities were found:

1) from phenotypes of infiltrates reported by Zeromski *et al*, (1987)

2) between motile cells and infiltrating cells.

In these laryngeal tumours several agreements with the work of Zeromski's group were found:

1) that tumour parenchyma was infiltrated by helper/inducer T cells.

2) that the peri-tumour was infiltrated by macrophages and CD4 T cells.

However, the infiltrates in the tumour parenchyma and peri-tumour were more variable in my study. In one case (case 1) there was infiltration by macrophages and helper/inducer T cells in tumour parenchyma, which were also found in peritumour in addition to Pan T cells and cytotoxic/suppressor T cells. In case 2 the tumour was infiltrated by all those cells described in case 1 with the addition of B cells and TCRδ-1 cells. These results indicated that motile cells could be of a greater variety than expected from the results of Zeromski's group, and extra markers were employed to identify motile cells accordingly. A further difference found was that no NK cells were detected in my study; but were found by Zeromski *et al*.

Comparing the phenotypes of motile cells with the infiltrates in sections has shown that macrophages and B cells were not motile on cultures, although present in sections. This suggested that there may be an inhibitor of macrophage and B cell motility present in the culture system. This was not further investigated in this study.

Immunofluorescence methods did not lead to identification of positive cells. This may have been because paraformaldehyde fixative was used which may have been too mild to permeabilise keratinocytes.

Identification of Motile Cells

The identification of motile cells was difficult for several reasons:
1) some motile cells were dislodged from the culture,
2) many motile cells either did not stain or stained negatively,
3) non-motile positive cells were present in the vicinity of the motile cells. This emphasised the need to be precise when tracking cells,
4) the immunofluorescence technique failed to identify motile cells.

However, the methods of PAP and APAAP identified several motile cells in cultured tumour and true cord. All motile cells, whether positively or negatively stained, were assessed for their morphology. This showed conclusively that all motile cells were of lymphocytic morphology. Motility was demonstrated for Pan T cells, helper/inducer T cells and suppressor/cytotoxic T cells. Other motile cells which were found were null cells and HML-1 cells.

There were only two types of cell motile on tumour cultures. These were Pan T cells and suppressor/cytotoxic T cells. The Pan T cells were

not further identified to assess phenotype, but as suppressor/cytotoxic T cells were also identified but not helper/inducer T cells, it is highly unlikely that helper/inducer T cells were motile on tumour cultures. This is an unexpected finding for several reasons. Firstly sectioned laryngeal tumour was found to have an infiltration of CD4+ macrophages and helper/inducer T cells within the tumour and especially at the peri-tumour region. This was seen to be a mixture of macrophages and helper/inducer T cells upon comparison of cell size in stained sections. Secondly helper/inducer T cells from peripheral blood of cancer patients were found to be more frequently motile than cytotoxic/suppressor T cells from the same source by Ratner *et al*, (1985). However there is evidence that in laryngel carcinoma there is a decrease in the numbers of CD4 T cells infiltrating tumour in the advanced stages of the disease (Zeromski *et al*, 1988). The implications of the results in this thesis in context with the results of other workers is that motility of helper/inducer cells is impaired. This may be due to the presence of an inhibitor or low cell numbers. The significance of this would be that the immune regulation of suppressor/cytotoxic T cells by helper/inducer T cells would be impaired and allow the tumour to proceed unchecked.

Normal laryngeal cultures were found to have a greater heterogeneity of motile subset type than tumour cultures. All cells were T cells. The majority were suppressor/cytotoxic T cells, although helper/inducer T cells were detected. A ratio of 3 to 1 cells was found. No macrophages were found to be motile. These findings suggest that normal cultures reflected the more normal immune status of this tissue compared to tumour.

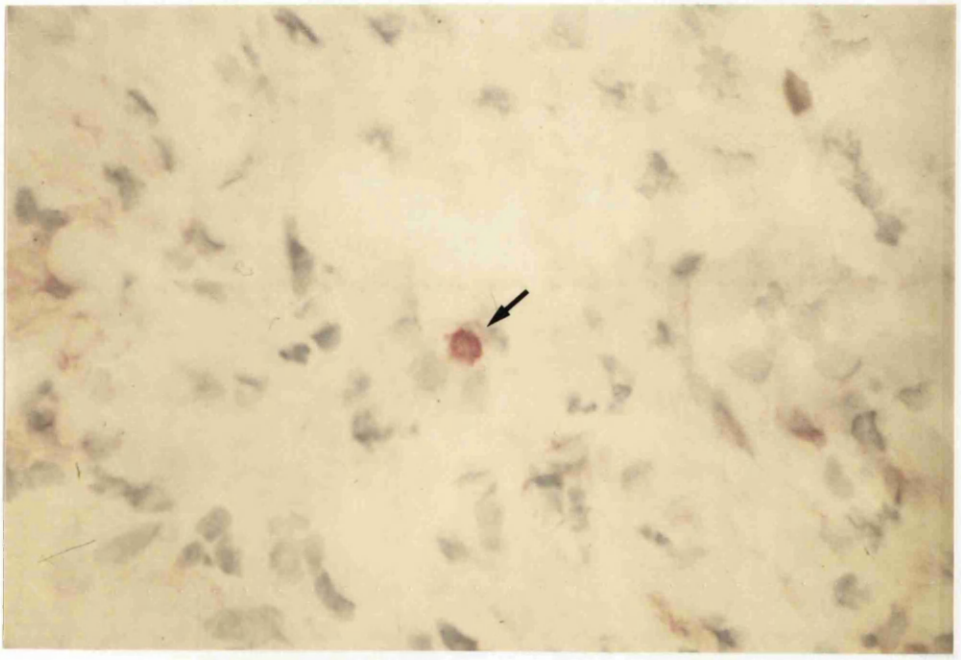
Absence of Motile Macrophages

The fact that no motile macrophages were identified on tumour or normal cultures and that all motile cells were lymphoid is of interest. This is a surprising finding since IL-1, a stimulant responsible for macrophage activation, is known to be produced by T lymphocytes. As described already in this chapter the predominant infiltrating cells in tumour stroma are T lymphocytes and macrophages. However, there are very few of these cells within the tumour *per se*. Thus, from whichever source motile cells are derived (tumour-associated tissue or tumour blood vessels) it is evident that the motile macrophage population is missing. There are several possible explanations for this. Firstly, there may be an inhibitor of macrophage locomotion present in the environment, such as interferons which tend to be growth-inhibitory (Trinchieri & Perussia, 1985). My results fit well into the findings of Balm *et al*, (1982) who looked at peripheral blood monocytes from patients with laryngeal cancer and found that this cancer had monocytes with poor migratory ability. Their work lacked a model for demonstrating the activity of monocytes and macrophages from the tumour region. The conclusion from the results of my study and those of Balm *et al* is that migration of macrophages in laryngeal cancer is specifically impaired in *in vitro* tests. If this occurs *in vivo* this may be a significant factor in the failure of these mature macrophages to infiltrate in large numbers into the tumour parenchyma and serve a tumour-killing role. It suggests that the tumour-cytotoxicity of macrophages is intrinsically-linked to their motility. An alternative explanation of this is that macrophages are not attracted into the deeper regions of the tumour *per se*.

Absence of Natural Killer Cell Infiltrates

The accumulation of macrophages in the peri-tumour region may prevent further anti-tumour activity. Macrophages have an inhibitory effect on natural killer (NK) cells. This is because macrophages produce prostaglandin E2 (PGE2), which inhibits NK cells (Balch *et al*, 1982). This supports the findings presented in this thesis, which did not detect NK cells as infiltrates or motile beneath culture. This may also support the findings of Schantz *et al*, (1987) who found that patients with head and neck cancer showed reduced NK cell function compared to healthy controls. These are potent killer cells because they do not require the presence of major histocompatibility antigen to function. Another killer cell which is non-restricted is the double negative CD4-, CD8-, CD3+ T cell. No motile cells were identified as killer cells from either of these two groups. However there was a positive result for the double negative T cell in one tumour section (Fig 6.13). In general there were no killer cells detected in sectioned tumour or present in motile form beneath the culture. This may be an important factor in the aetiology and progression of laryngeal carcinoma.

Fig 6.13 Laryngeal tumour section showing a positive TCRδ-1 cell ($CD3^+$,$CD4^-$,$CD8^-$) stained with monoclonal antibody TCRδ-1 using APAAP technique and fast red chromagen. Magnification x720



Cytotoxic T Cells Cannot be Distinguished from Suppressor T Cells by Staining

If stained, the identity of a motile cell could not always be definitively linked to phenotype function. For example, monoclonal antibody UCHT4 could not distinguish between cytotoxic and suppressor T cells, because these cells bear the same CD8 surface antigen. A monoclonal antibody Leu 15 has been described which was purported to identify suppressor cells but was unsuitable for use in this study, because the antigen was unstable after 8 hours. Tracking and fixation of cultures usually took longer than 8 hours. Furthermore it also labels some macrophages and NK cells, so that sequential staining was required. Sequential staining procedures were attempted, but it was found that cultures were easily damaged and therefore they were not continued.

Specificities of Leu3a Cells

Another example in which it was hoped that sequential staining might aid phenotype identification was in the identification of the two types of Leu3a cells. These had in common the surface antigen CD4, which was present on helper/inducer T cells and some macrophages. Sequential stains used in this study failed to show if the cells were helper/inducer T cells or macrophages.

Therefore the morphology of all CD4+ cells on cultures was checked retrospectively as a complete collection of cultures. This was carried out on all areas of culture despite the lack of time-lapse recording for most areas. Most CD4+ cells were lymphocytes, except in one culture where there were many lymphocytes and also cells with macrophage morphologies; having

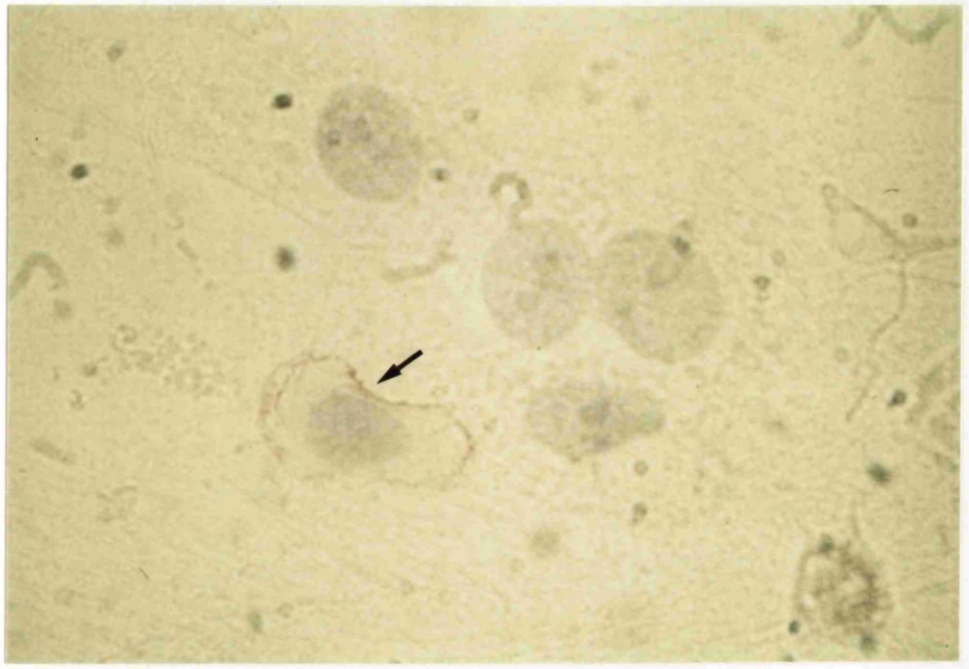


Fig 6.14 Non-neoplastic culture with very large Leu3a positive cell stained by APAAP technique with fast red chromagen and Leu3a monoclonal antibody. Counterstained with haematoxylin. This may be a macrophage. Magnification x3600

Fig 6.15 Scanning electron micrograph of non-neoplastic culture with macrophage-like cell (M) and lymphocyte (L). Magnification x 6703

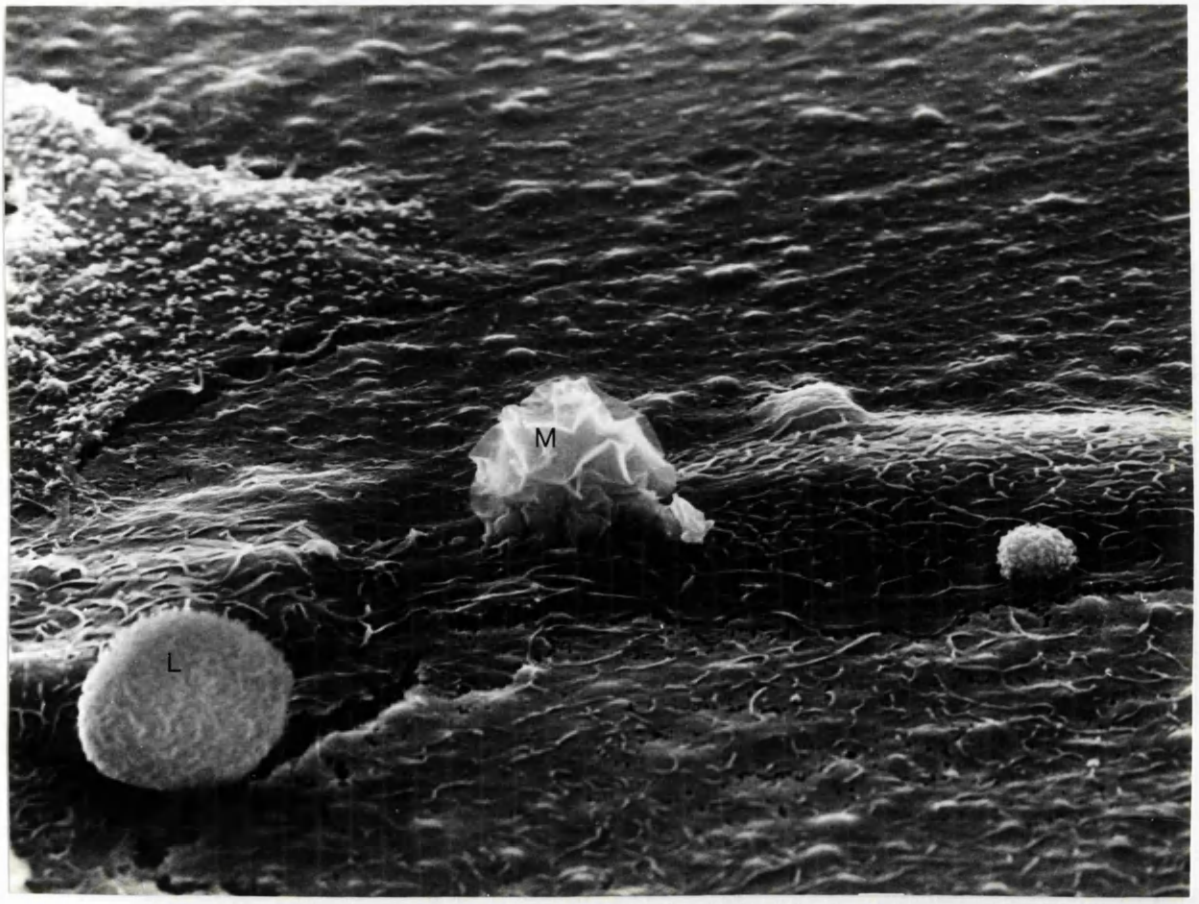


Fig 6.16 Scanning electron micrograph of non-neoplastic culture with macrophage-like cell. Magnification x 4128

more cytoplasm than lymphocytes. A typical macrophage on this latter culture is presented in Figure 6.14, and also as seen in other cultures by SEM (Fig 6.15 and Fig 6.16) which may or may not have been motile. The morphology of both these macrophages suggests that the cells are in the motile form. Although immunocytochemical staining with specific monoclonal antibodies for macrophages was carried out, no macrophages were identified as motile cells.

Problems with Staining of Lymphocytes

It became obvious that many motile cells were not staining. This problem was investigated to assess whether:

1) they did not stain because they genuinely did not express the antigen under test, or

2) because the antibody did not penetrate the monolayer.

Monoclonal antibodies would gain access beneath cultures via microscopical holes in cultured cells formed by permeabilisation. Adequate permeabilisation would be essential since antibodies are very large molecules. The method of acetone fixation was assessed and found to be the optimum agent for permeabilisation of cultured cells for immunostaining. This was supported by the finding of positively stained basolaterally-situated cells. However the patchy keratin distribution may have importance in this problem, since keratinised cells might need extra permeabilisation methods, comprising freeze-thaw techniques and the use of detergents. Sites of keratinisation may prevent lymphocytes from staining. This is a possible explanation for the failure of this method to stain the majority of lymphocytes, although the method occasionally produced very good results.

Examination of laryngeal cultures stained by Leishman stain demonstrated that, in some, there are many holes in the culture. Access of antibody at these cells might well be enhanced. This was a further reason to explain differential staining results.

Infiltrates Within Explants

Explants were found to have vessels containing cells which labelled with a variety of lymphoid markers. These vessels also contained red blood cells and therefore were blood vessels. Lymphocytes and monocytes present in the tumour blood vessels may be stimulated by factors derived from the surrounding medium and proliferating monolayer. During incubation many of these cells would become activated and therefore motile. There would presumably be some autolysis as the explant tissue began to decay, which would expose the vessel contents and enable motile cells to move from vessel to surrounding tissue and eventually out towards the culture. This suggests two facts. Firstly it suggests that the lymphocytes survive well in vessels and secondly that they are resilient enough to withstand autolytic enzymes which break down the vessel wall. There may of course be a simpler explanation that is based on extravasation or a similar phenomenon.

Significance of HLA-DR Antigen

In explants, the cells contained within carcinoma vasculature and the vasculature itself stained positively for HLA-DR antigen (Fig 6.6). This finding suggests that in laryngeal carcinoma, lymphocytes would be stimulated by their natural environment. Further work is required to evaluate the number of motile cells which express HLA-DR antigen, and also

whether cultured carcinoma itself expresses this antigen. This would be interesting to assess since Esteban *et al,* (1989) have correlated HLA-DR antigen expression in laryngeal squamous cell carcinoma with a more favourable prognosis. Since HLA-DR antigen is important in attracting and activating cytotoxic T lymphocytes it may be a chemotactic influence in this model of lymphocyte locomotion. Alternatively if cultures do not express HLA-DR antigen this may explain why the carcinoma has progressed despite the presence of suppressor/cytotoxic T lymphocytes. Lack of HLA-DR antigen expression in laryngeal carcinoma cultures may explain the failure of motile T cells to be cytotoxic on cultures.

The Source of Motile Lymphocytes

The precise source of the motile population of lymphocytes observed on cultures cannot be derived from this study. However it can be inferred that there are three possible sources. These are in particular the stroma and blood vessels and to a lesser extent the parenchyma. The periphery of each tumour explant would release locally infiltrating lymphocytes, which were found accumulated in stromal regions. If these cells are in an activated form, it would be possible for locomotion to occur immediately. However, lymphocytes do not adhere or move on plastic substrata. Therefore these cells would be observed to move as soon as the first progeny tumour cells were produced, since they require a monolayer for locomotion. This agrees with the finding of Haston (1979) and Ager (1988) who demonstrated that lymphocytes need to adhere before moving and indeed to move preferentially beneath monolayer cells.

CHAPTER 7

VECTOR ANALYSIS OF LYMPHOCYTE LOCOMOTION

VECTOR ANALYSIS OF LYMPHOCYTE LOCOMOTION

INTRODUCTION

This chapter deals with the interpretation of lymphocyte locomotion on laryngeal cultures as observed by video time-lapse microscopy. Vectors derived from observed pathways will be compared statistically to examine whether the apparent persistent movement of lymphocytes towards some mitotic cells of the monolayer is truly directional. Such an analysis is necessary because lymphocyte locomotion may be random motion (kinesis or guidance) or non-random (taxis). Taxis has been described as a vectorial movement (i.e. having magnitude and direction); whereas a kinesis is a scalar movement (i.e. having only magnitude not direction) Dunn, (1981). A visual study comparable to this part of the thesis of lymphocyte taxis has been described by Wilkinson (1985). He employed lymphocytes separated from peripheral blood and applied a chemotactic agent while filming locomotion in collagen gels by time-lapse. Although this method is the most direct way to demonstrate locomotion, it was not definitive for taxis until other types of locomotion had been considered and excluded. For this purpose lymphocyte pathways were analysed as vectors. This method of vector analysis will also be used in this thesis and is based on the work of Dunn (1981), which looks at directed cell behaviour. The theme of this chapter is to test the vectors of laryngeal lymphocytes moving in the vicinity of mitotic cells of laryngeal tumour and normal epithelial culture for taxis.

METHOD

Video Description

Four video tapes were selected for study, as follows:

Tumour 1: Single lymphocyte moving on tumour culture towards mitoses occurring in a colony of proliferating tumour cells.

Tumour 2: Several lymphocytes moving within a 360 degree zone around mitoses in tumour culture.

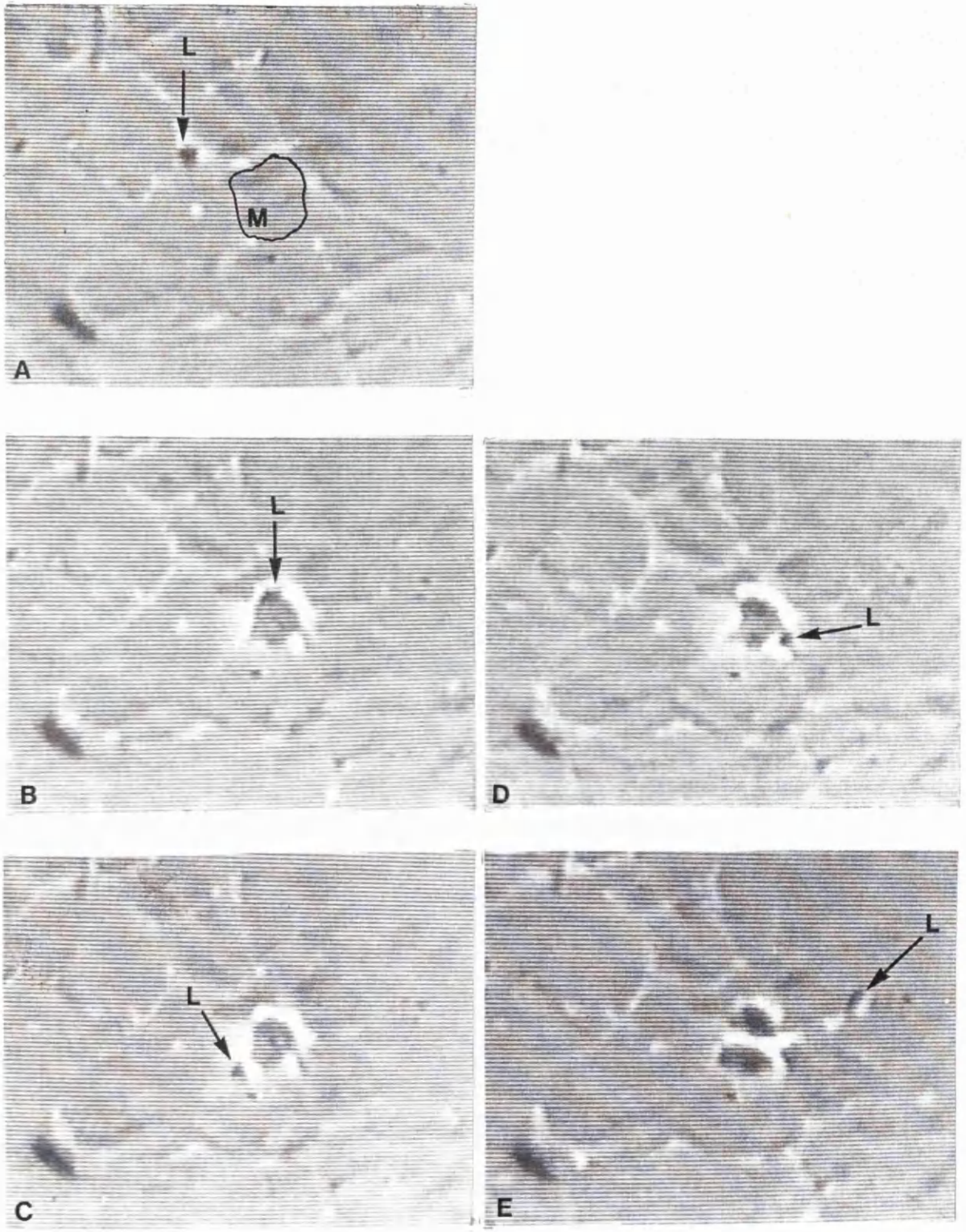
Normal 1: Single lymphocyte moving in the vicinity of mitoses in normal true cord culture. Still shots are shown in Figure 7.1.

Control: Several lymphocytes moving apparently at random in culture of normal aryepiglottic fold , which did not have mitotic cells.

Displacements

Sequences of lymphocyte displacement recorded at intervals of 10 s were reviewed. Calibration of the screen was carried out using a graticule displayed onto the monitor. This was necessary to measure the distances between lymphocytes and mitoses. An acetate sheet was fixed to the screen of the monitor. The centre of each motile lymphocyte was dotted at one minute time intervals. Dots were joined by straight lines to display pathways, of which examples were drawn on paper (see Figure 7.2 in results section of this chapter). Consecutive 1 min displacements were grouped into series of five minute displacements. Each 5 min displacement was coded as follows. Each lymphocyte was given a capital letter, identifying the nearest mitotic cell and each 5 min displacement was given a number. Thus, a single lymphocyte pathway of 20 minutes near mitotic cell 'A' would

Fig 7.1 Outgrowth from true cord (A). In the centre there is an epithelial cell (M) entering mitosis and an approaching lymphocyte (L). The lymphocyte circumnavigates the cell in mitosis twice (B-D) and finally it is leaving the site of mitosis (E).



have 5 minute displacements A1, A2, A3 and A4. In the case of more than one lymphocyte approaching the same mitotic cell, displacements were given an initial denoting individual lymphocytes. For example, Na1 and Na2 are the first and second 5 minute displacements of lymphocyte 'a' approaching mitosis 'N'. Each cell was given coordinates in relation to an x axis journey, the starting point of the lymphocyte at (0,0) to the mitotic cell and a y axis with the same origin but at 90 degrees to the x axis. These coordinates represented magnitude and direction of displacement, and therefore constituted vectors.

Using Vectors

The simplest evidence of non-randomness i.e. taxis, would be that there was a significant mean cell displacement along the x axis. An approach to testing for the significance of this mean would be to consider all the individual displacements joined to form a 'walk'. In a random walk the net displacement of steps would be close to the origin, because each step differs in direction from the next so that there is no net advancement along the x axis. The order of steps in a walk is irrelevant to the net displacement, because each displacement starts at the origin. It is possible to formulate a null hypothesis that the net displacement of a random walk is no greater than the net displacement of a pure random walk in two directions i.e. not only towards the x axis, but to any point in a 360 degree field. Thus a directed walk would have a net displacement which is well-displaced from the origin and thus exceeds the net displacement of the null hypothesis. The easiest way to test this hypothesis is to construct a vector scatter diagram. A dot was placed marking the x and y coordinates where each displacement ended. In the case of a positive taxis, the mean of the x coordinates would be positive, i.e. in the direction of

the mitotic cell. A mean situated at zero would indicate random movement. The mean y coordinate (i.e. displacement at 90 degrees to any tactic influence deriving from the mitotic cell) should not differ significantly from zero, thus producing an internal control demonstrating the absence of any other directional influences.

Statistical Analysis of Vectors to Test for Non-randomness

The arithmetic mean and standard deviation of the mean displacement in the x and y axes were calculated separately, using the formula:

$$s=\sqrt{\frac{\epsilon\ (x-X)^2}{n-1}}$$

where s = standard deviation

x = individual score

X = mean score

ϵ = 'the sum of'

Median rank test confirmed that the data was normally distributed. Therefore the application of student's t test was appropriate. Student's t-test was used to calculate the t value, using the following formula:

$$t=\frac{|x-\mu|\sqrt{n}}{s}$$

where x = arithmetic mean of coordinates

μ = zero (null hypothesis)

n = number of displacements

s = standard deviation

The t value was compared to the table t value at the appropriate number of degrees of freedom at the 0.05 significance level (p=0.05). This level of significance is generally used in biological statistical calculations. If, for example the t value were greater in magnitude than

the table t value, this would indicate that the data does not agree with the null hypothesis $\mu=o$. The null hypothesis in this chapter assumes that if displacements are distributed randomly in all directions then the net displacement is approximately equivalent to zero. In this example the mean displacement would be consistently in the direction of the mitotic cell and demonstrates non-random locomotion. Conversely, if the t value were less than the table t value, this would indicate that the null hypothesis was correct, so that the locomotion would be predominantly random.

T-tests were carried out on displacements in the following populations:

1) Lymphocytes near mitoses in tumour culture 1. Displacements were designated D to M.

2) Lymphocytes near mitoses in tumour culture 2. Displacements were designated N to R.

3) Lymphocytes near mitoses in normal culture. Displacements were designated A to C.

4) Lymphocytes in normal culture without mitoses. Displacements were marked with an asterisk.

Each of groups 1-3 was subdivided according to the mitotic phase of the nearest mitotic cell (i.e. metaphase to mid-anaphase or mid-anaphase to telophase). A cell in metaphase was identified by the condensing of chromosomes and the rounding of the cell. Mid-anaphase appeared as chromatids separated to opposite poles. Telophase was the point at which the daughter cells separated.

Preliminary observations suggested that the mitotic cell did not influence lymphocyte movement at a distance of 200 μm. Therefore, each of the above groups was sub-divided according to the distance of each

displacement from the mitotic cell i.e. a zone of radius 0-100 μm; a zone of 100-200 μm and a zone of 200 μm to the rectangular border of the monitor. The maximium distance was approximately 220 μm. Lymphocyte displacements were grouped in this way to test for the effect of distance on direction of locomotion.

Displacements in population 4 were divided into displacements in relation to an arbitrary point in the culture at the same distances as above, occurring during 30 mins, i.e. the time for mitosis to progress from metaphase to telophase.

RESULTS

Some but not all lymphocyte pathways are presented in Figs 7.2 i-iv (tumour 2). Vector scatter diagrams are presented in Figs 7.3 i-iii. The x and y coordinates of vectors are shown in Tables 7.1-7.7. Results of t-tests are shown in Tables 7.8 i-iv and summarised in Tables 7.9 i and ii.

Vector scatter diagrams (Figs 7.3.i-iii) show plots of individual vectors and also mean lymphocyte displacement for each population. The simplest way to assess if taxis has taken place is to consider the position of vectors and the mean displacement. In the case of taxis the mean displacement along the x axis should be towards the attractant M, while control mean displacements should be close to the origin, indicating random walks. A mean displacement at considerable distance from the origin, indicating taxis, can be seen for both tumour 1 and tumour 2 in zones 1 and 2 (0-200 μm) during metaphase to mid-anaphase. Student's t test applied to the x coordinates confirmed that the responses were taxes. This was not so for the same lymphocytes in the same zones during the mitotic stages of mid-anaphase to telophase. Taxis though less marked, was found in the normal laryngeal culture during metaphase and anaphase. The change in response of lymphocyte locomotion from taxis to a random walk was shown by vector analysis to occur in synchrony with separation of daughter nuclei. Vector analysis clearly shows that the lymphocytes which were near the mitosis prior to the separation of daughter nuclei, altered course significantly, no longer in the direction of the mitotic cell and always the mean displacement was close to the origin. Student's t-test applied to the y coordinates always indicated a random response, suggesting that the system was free of tactic influences; other than those originally from the mitotic cells.

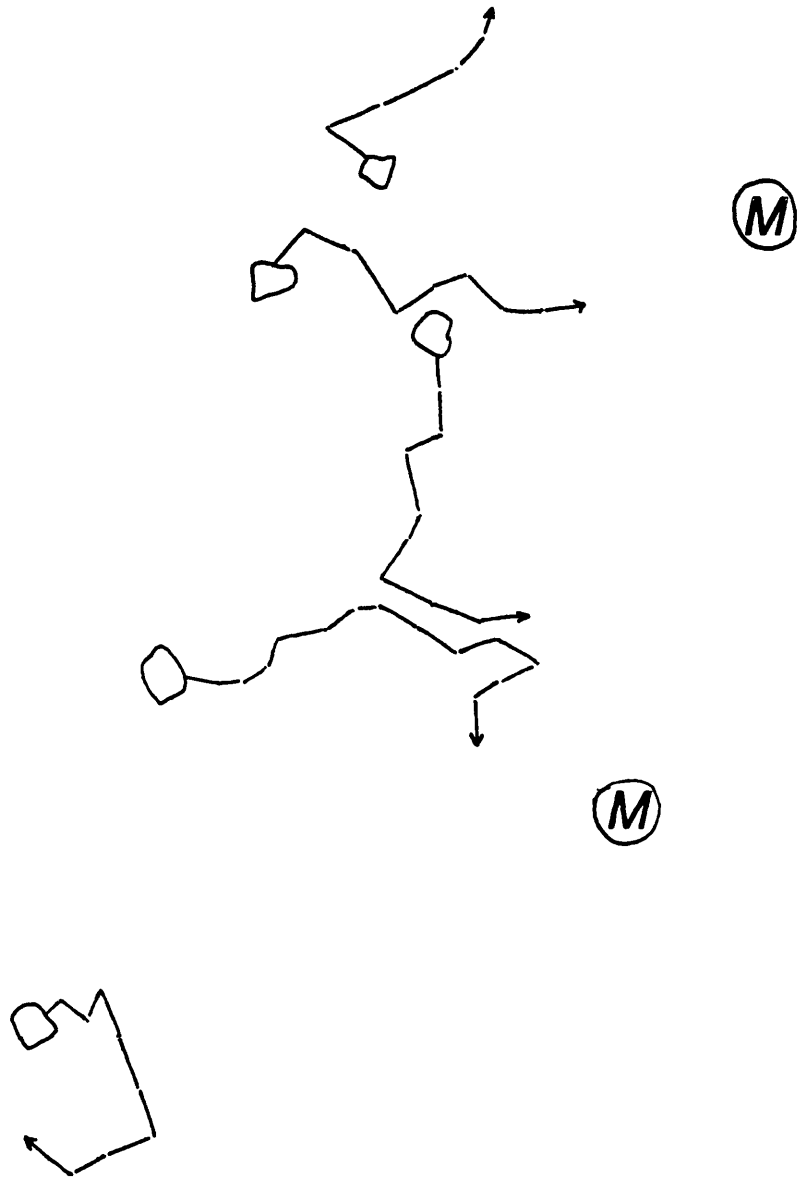
In tumour 1 Fig 7.3.i b) the mean displacement during metaphase to mid-anaphase is close to the origin but lies to the right of the y-axis and therefore it is not easy to interpret exactly what this represents from the scatter diagram alone. Student's t-test gave a t value of 2.54 which is very close to the corresponding table value of 2.15, i.e. this is a borderline result. The data for this population was composed of 15 displacements of 4 lymphocytes in relation to 10 mitoses. This number of mitotic cells emitting attractants at different times could well have created this effect.

Evidence of taxis towards mitosis was found in all tests within 200 μm of each mitosis during the mitotic stages of metaphase until mid-anaphase. No difference was found between results of lymphocytes on tumour and normal cultures. Most lymphocytes changed course as soon as daughter nuclei of dividing cells separated, as demonstrated on video at time code 00:04:37. Vector analysis of these latter displacements confirms that locomotion changed rapidly from a taxis to a random form which continued during telophase. The control population of lymphocytes (i.e. moving in the absence of any mitosis), showed evidence of similar random locomotion at all times and in all situations.

At distances of greater than 200 μm (zone 3), mean x displacement was random for all groups during all stages of mitosis.

Fig 7.2 i) Lymphocyte pathways from tumour 2.

throughout metaphase, anaphase and telophase.



M = mitosis

Fig 7.2 ii) Lymphocyte pathways from tumour 2

throughout metaphase, anaphase and telophase.

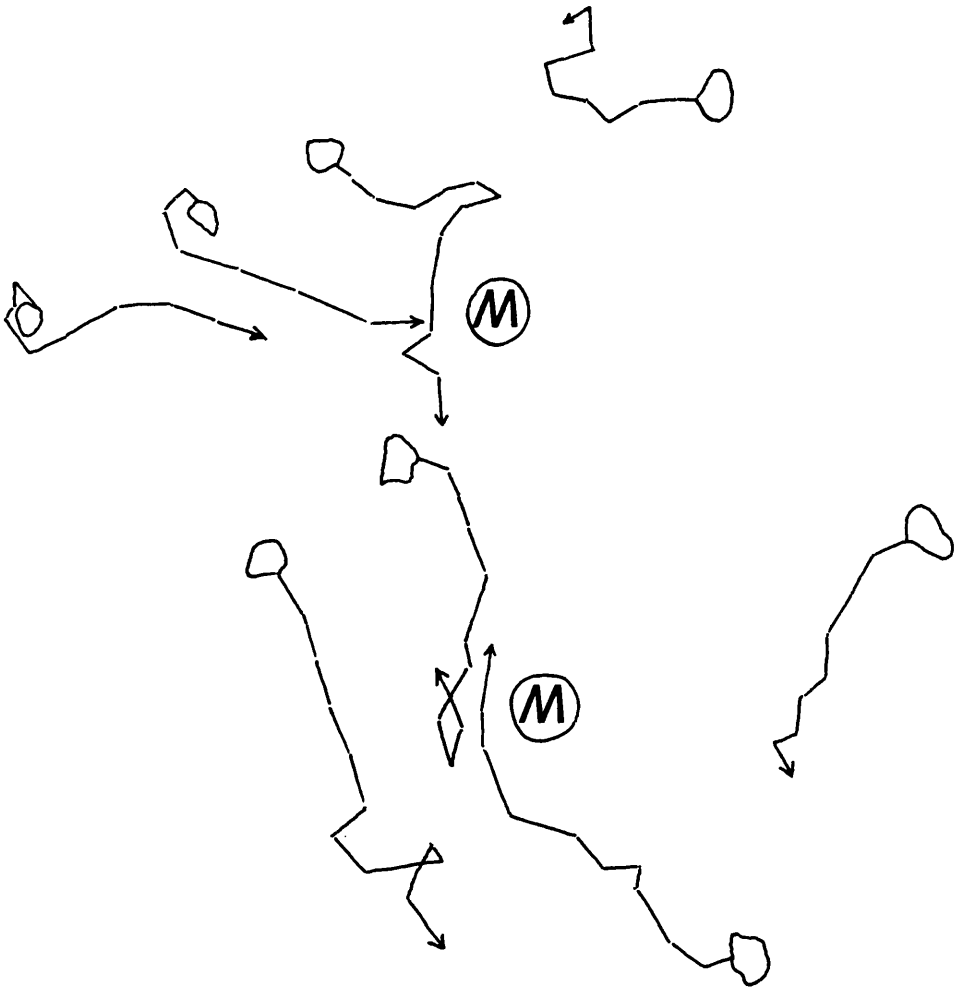


Fig 7.2 iii) Lymphocyte pathways from tumour 2

throughout metaphase, anaphase and telophase.

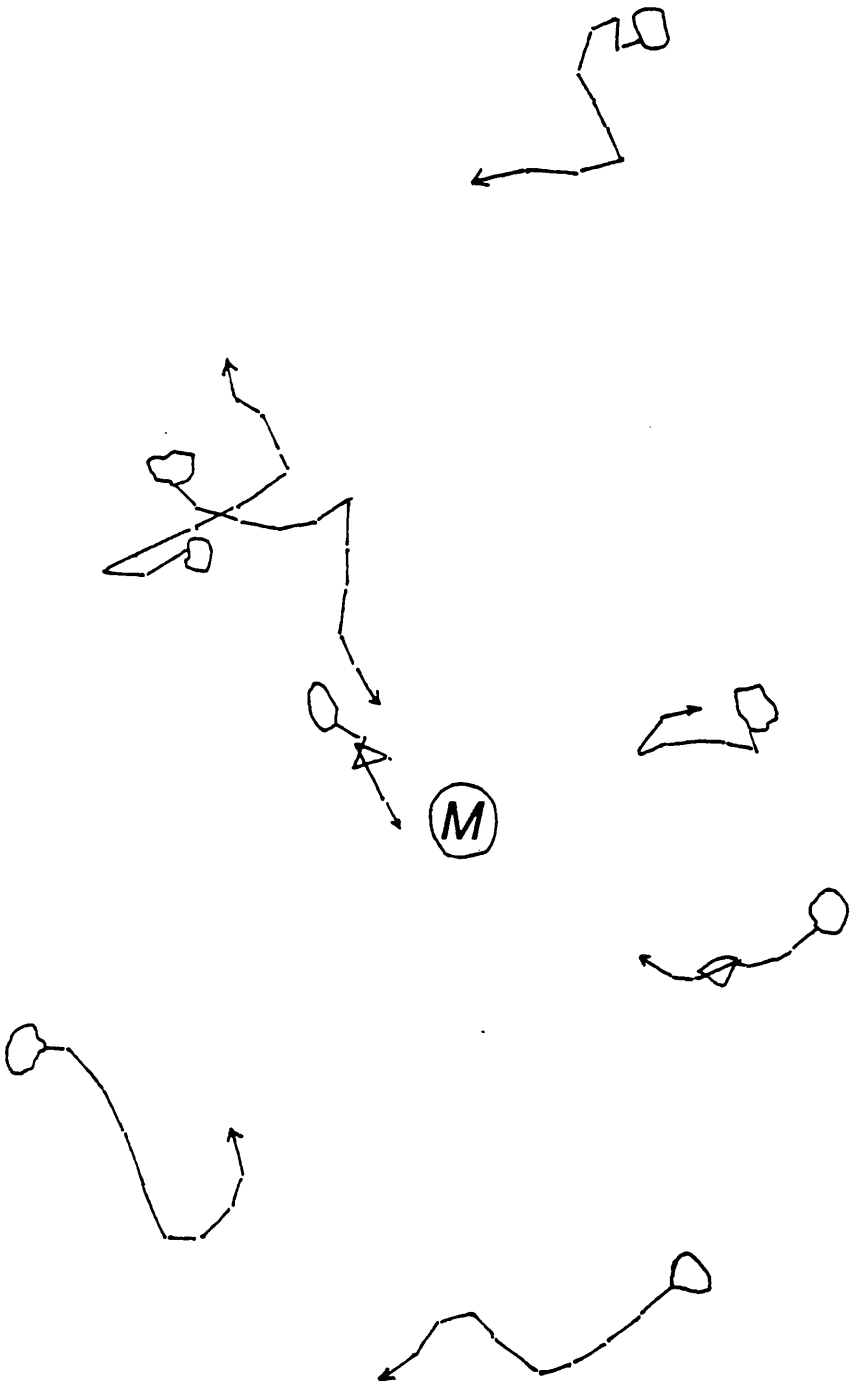


Fig 7.2 iv) Lymphocyte pathways from tumour 2

throughout metaphase, anaphase and telophase.

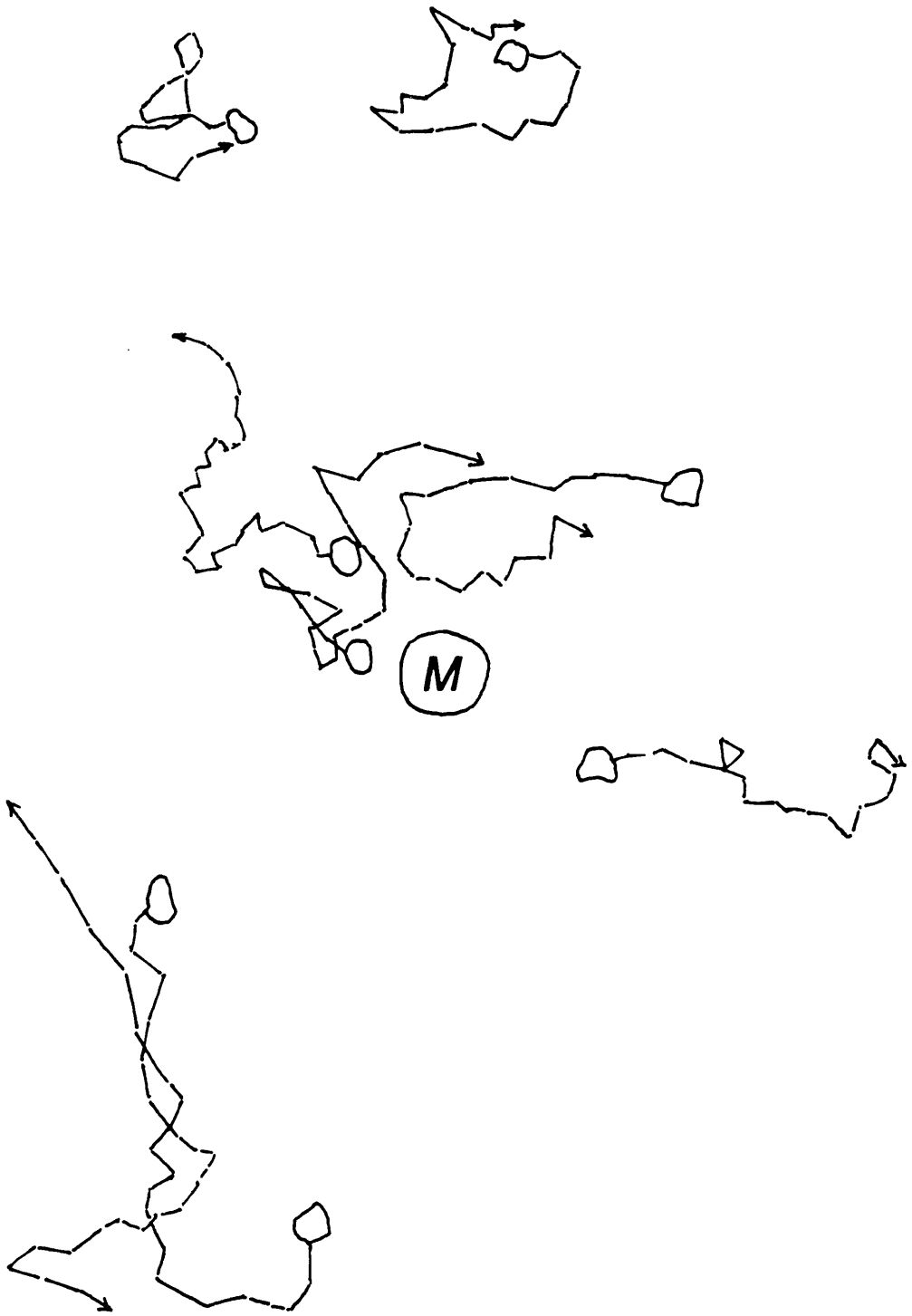


TABLE 7.1

TUMOUR 1. VECTOR CO-ORDINATES OF 5 MINUTE DISPLACEMENTS OCCURRING DURING METAPHASE TO MID-ANAPHASE OF MITOSES D-M

ZONE 1 (0-100 μm)			ZONE 2 (100-200 μm)			ZONE 3 (>200 μm)		
DISPLACEMENT	X	Y	DISPLACEMENT	X	Y	DISPLACEMENT	X	Y
E1	15	-1	D1	8	-11	I2	9	-11
E2	11	-8	D2	11	-5	I3	17	-3
E3	16	9	D3	14	3	I4	6	8
G1	14	-5	F3	11	5	I5	-7	7
L5	13	-3	F4	17	14			
L6	16	-1	J5	23	-9			
M1	-9	7	J6	16	-15			
M2	5	-3	K2	13	-3			
M3	-4	-5	K3	4	-6			
			K4	2	9			
			L1	10	-6			
			L2	10	-13			
			L3	12	0			
			L4	11	3			

TABLE 7.2

TUMOUR 1. VECTOR COORDINATES OF 5 MINUTE DISPLACEMENTS OCCURRING DURING MID-ANAPHASE TO TELOPHASE OF MITOSES D-M.

ZONE 1 (0-100 μm)			ZONE 2 (100-200 μm)			ZONE 3 (>200 μm)		
DISPLACEMENT	X	Y	DISPLACEMENT	X	Y	DISPLACEMENT	X	Y
D4	7	13	J1	-25	-9	I2	-1	13
D5	-3	-5	K1	-10	15	I3	-14	-5
E4	-11	2				J3	2	4
E5	4	2				J4	1	13
F1	22	7						
F2	15	-9						
G2	6	7						
H1	8	-13						
H2	15	-4						
L7	10	10						
L8	-13	3						
M4	5	-5						
M5	4	4						
M6	9	-10						
M7	17	7						

TABLE 7.3

TUMOUR 2. VECTOR COORDINATES OF 5 MINUTE DISPLACEMENTS OCCURRING DURING METAPHASE TO MID-ANAPHASE OF MITOSES N,O,P,Q & R

ZONE 1 (1-100 μm)			ZONE 2 (100-200 μm)			ZONE 3 (>200 μm)		
DISPLACEMENT	X	Y	DISPLACEMENT	X	Y	DISPLACEMENT	X	Y
Nc1	13	10	Na1	5	-29	Nh1	14	-4
Nc2	8	2	Nb1	4	27			
Nd1	13	-3	Nf1	1	10			
Ne1	11	-3	Nf2	-18	8			
Ng2	28	-4	Ng1	18	16			
Oa1	23	-27	Pa1	6	-2			
Oa2	6	-21	Qa1	11	-12			
Ob1	25	-2	Qb1	21	17			
Ob2	2	-9	Qc1	23	-13			
Oc1	22	6	Ra1	30	-8			
Oc2	32	2	Rb1	12	19			
Od1	23	12						
Pa2	25	-2						
Pb1	-4	-2						
Pb2	35	0						
Pc1	17	9						
Pc2	18	-10						
Pd1	12	-15						
Qb2	21	4						
Qc2	21	10						

TABLE 7.4

TUMOUR 2. VECTOR COORDINATES OF 5 MINUTE DISPLACEMENTS OCCURRING DURING MID-ANAPHASE TO TELOPHASE OF MITOSIS N

ZONE 1 (0-100 μm)			ZONE 2 (100-200 μm)			ZONE 3 (>200 μm)		
DISPLACEMENT	X	Y	DISPLACEMENT	X	Y	DISPLACEMENT	X	Y
Nc3	-18	-11	Na2	-14	-13	Na5	-12	14
Nd2	14	-10	Na3	-8	-17	Na6	-2	-12
Nd3	5	-11	Na4	-11	0	Nb2	-13	20
Nd4	6	-11	Nb4	24	25	Nb3	17	18
Nd5	11	3	Nb5	0	22	Nf4	-4	-6
Nd6	-4	10	Nc4	-5	6	Nf5	-4	11
Nd7	-7	3	Nc5	-13	-2	Nf6	8	-5
Ne2	-3	5	Nc6	4	-10	Nh2	4	10
Ne3	4	-7	Nf3	-4	-6	Nh5	-14	6
Ne4	-3	24	Nf7	7	-9	Nh6	2	13
Ne5	2	19	Ng7	-15	1			
Ng3	-10	-6	Nh3	7	-11			
Ng4	0	-10	Nh4	0	-12			
Ng5	-8	9						
Ng6	2	12						

TABLE 7.5

NORMAL CULTURE 1. VECTOR COORDINATES OF 5 MINUTE DISPLACEMENTS OCCURRING DURING METAPHASE TO MID-ANAPHASE OF MITOSES A-C

ZONE 1 (0-100 μm)			ZONE 2 (100-200 μm)			ZONE 3 (>200 μm)		
DISPLACEMENT	X	Y	DISPLACEMENT	X	Y	DISPLACEMENT	X	Y
A1	7	-8	B3	-3	-3	NT		
A2	2	-6	B4	2	5			
A3	5	-7	B5	2	4			
A4	6	-6	B6	3	4			
A5	12	-8	B7	4	3			
A6	3	-8	C2	9	-9			
			C3	6	4			
			C4	4	5			

TABLE 7.6

NORMAL CULTURE 1. VECTOR COORDINATES OF 5 MINUTE DISPLACEMENTS OCCURRING DURING MID-ANAPHASE TO TELOPHASE OF MITOSES A-C

ZONE 1 (0-100 μm)			ZONE 2 (100-200 μm)			ZONE 3 (>200 μm)		
DISPLACEMENT	X	Y	DISPLACEMENT	X	Y	DISPLACEMENT	X	Y
A7	2	-5	C1	11	2	NT		
A8	-4	-6						
B1	7	5						
B2	8	2						

TABLE 7.7

CONTROL: NORMAL CULTURE 2 VECTOR COORDINATES OF 5 MINUTE DISPLACEMENTS OCCURRING RELATIVE TO A FIXED ARBITRARY POINT * IN CULTURE LACKING MITOSES

ZONE 1 (0-100 μm)			ZONE 2 (100-200 μm)			ZONE 3 (>200 μm)		
DISPLACEMENT	X	Y	DISPLACEMENT	X	Y	DISPLACEMENT	X	Y
*a1	18	-14	*b7	-11	5	NT		
*a2	11	-21	*b8	13	-3			
*a3	-14	-15	*b9	-4	-8			
*b1	19	17	*c2	-14	-3			
*b2	-13	3	*d1	10	-5			
*b3	3	-15	*d2	3	-12			
*b4	10	-11	*e2	-12	5			
*b5	8	-12	*e3	-11	12			
*b6	-14	-2	*f3	-9	5			
*c1	12	-9	*f4	-8	4			
*e1	2	19	*g1	13	2			
*f1	-6	0	*g2	20	7			
*f2	-2	5	*g6	-12	-3			
*g3	7	1	*g7	-19	-2			
*g4	9	-3	*h3	-9	-4			
*g5	-18	-8	*h4	1	5			
*h1	-6	-4	*h5	9	2			
*h2	4	-12						
*h6	4	8						

FIG 7.3.i VECTOR SCATTER DIAGRAMS SHOWING DISPLACEMENTS OF LYMPHOCYTES ON a) CONTROL, b) TUMOUR 1, c) TUMOUR 2, AND d) NORMAL CULTURES WITHIN ZONE 1 (0-100 μm) DURING METAPHASE TO MID-ANAPHASE AND MID-ANAPHASE TO TELOPHASE. THE CROSS SHOWS THE MEAN DISPLACEMENT FOR EACH LYMPHOCYTE POPULATION (p=0.05); M = MITOSIS

METAPHASE TO MID-ANAPHASE *MID-ANAPHASE TO TELOPHASE*

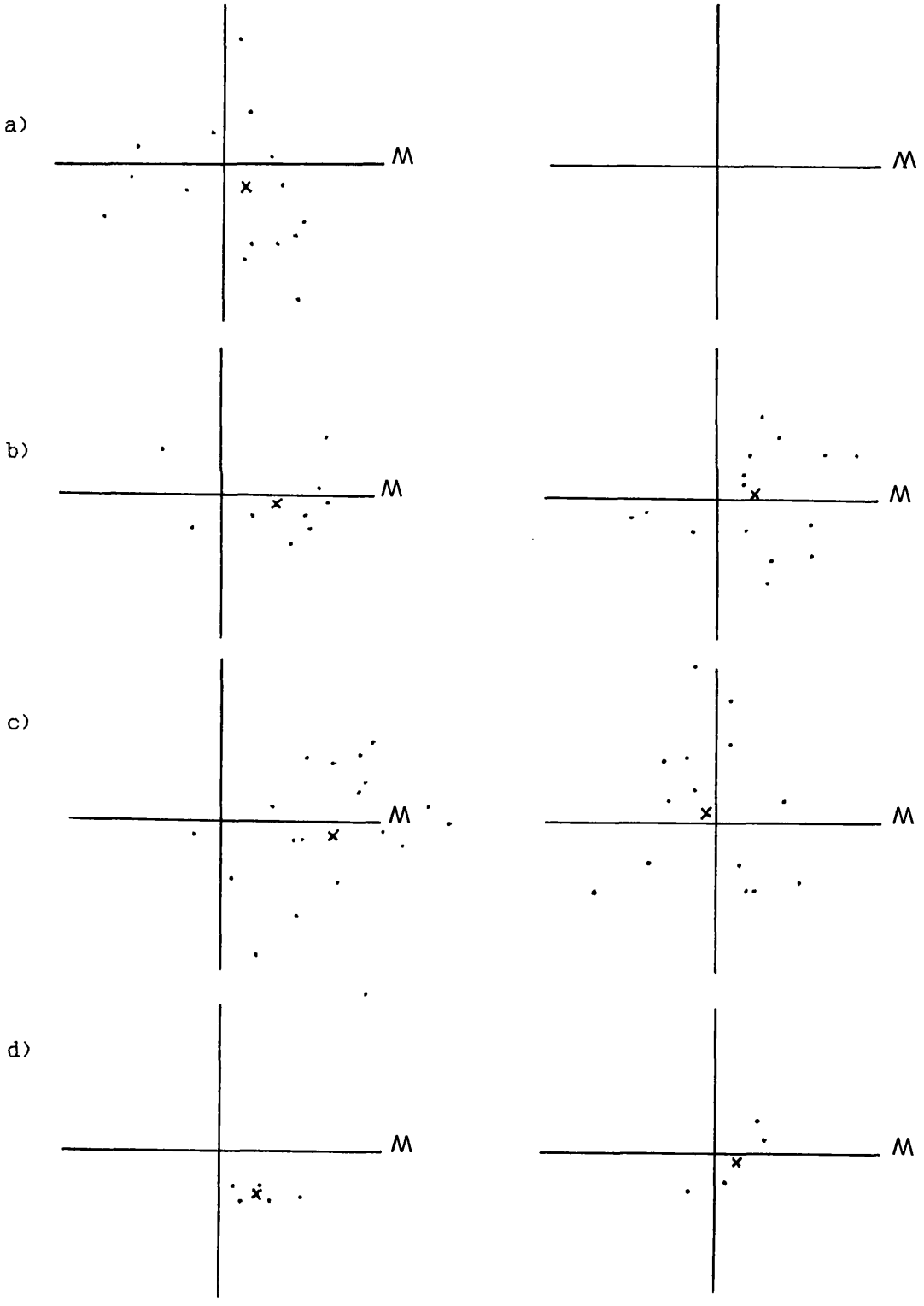


FIG 7.3.ii VECTOR SCATTER DIAGRAMS SHOWING DISPLACEMENTS OF LYMPHOCYTES ON a) CONTROL, b) TUMOUR 1, c) TUMOUR 2 AND d) NORMAL CULTURES WITHIN ZONE 2 (100-200 μm) DURING METAPHASE TO MID-ANAPHASE AND MID-ANAPHASE TO TELOPHASE. THE CROSS SHOWS THE MEAN DISPLACEMENT FOR EACH LYMPHOCYTE POPULATION (p=0.05); M = MITOSIS.

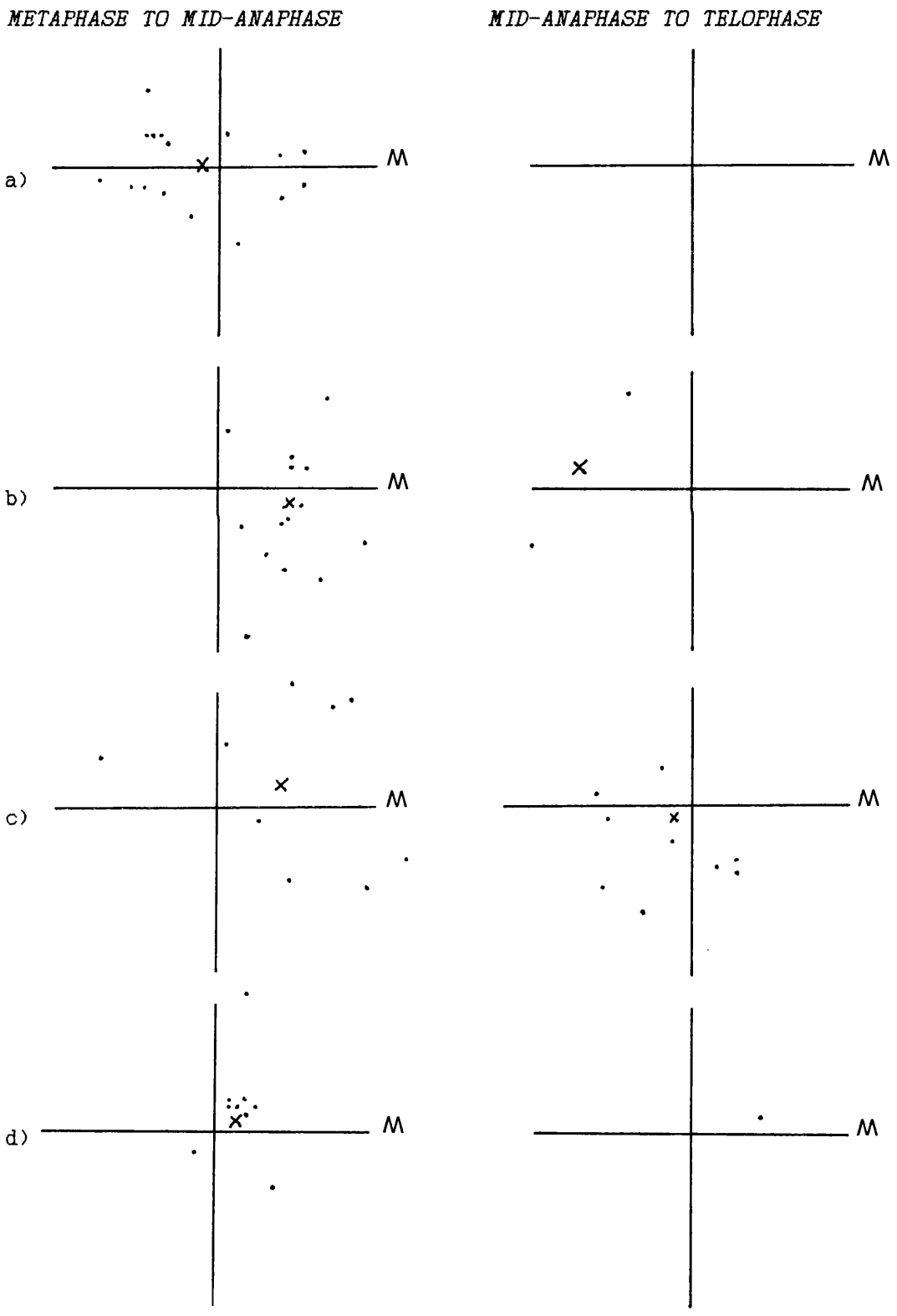


FIG 7.3.iii VECTOR SCATTER DIAGRAMS SHOWING DISPLACEMENTS OF LYMPHOCYTES ON a) TUMOUR 1 AND b) TUMOUR 2 WITHIN ZONE 3 (>200 μm) DURING METAPHASE TO MID-ANAPHASE AND MID-ANAPHASE TO TELOPHASE. THE CROSS SHOWS THE MEAN DISPLACEMENT FOR EACH LYMPHOCYTE POPULATION (p=0.05); M = MITOSIS

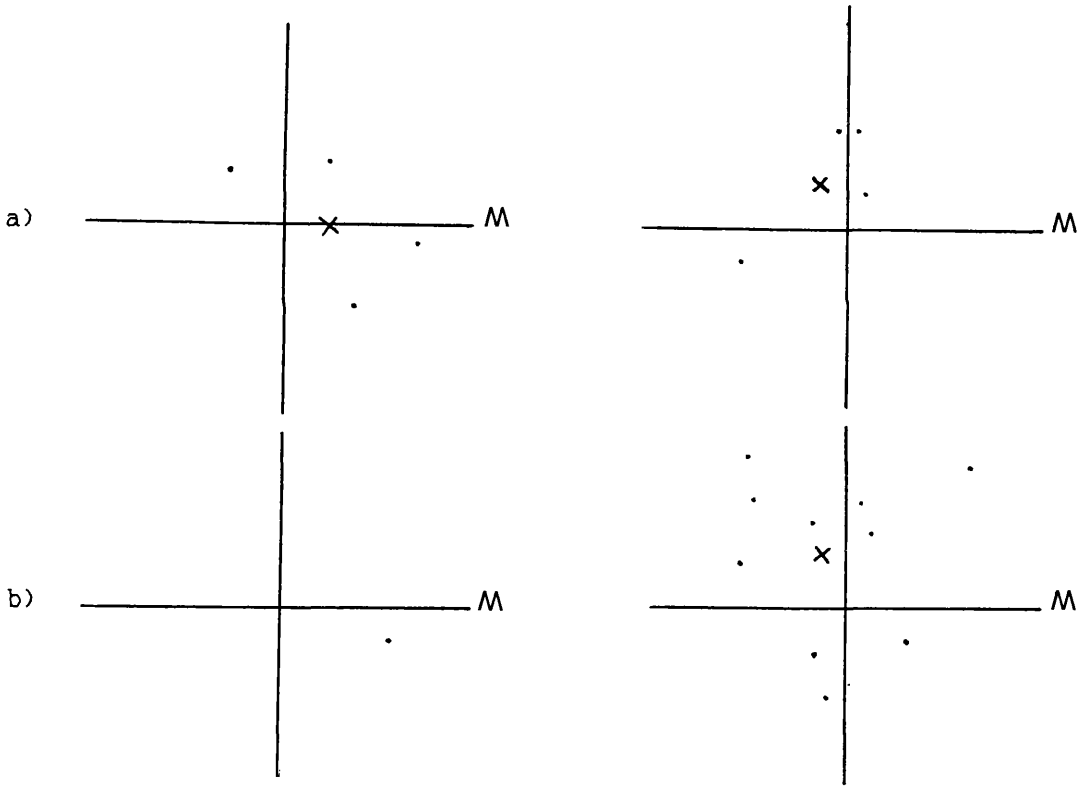


TABLE 7.8.1 STATISTICAL RESULTS OF MEAN X DISPLACEMENTS IN TUMOUR CULTURES

SAMPLE POPULATION	RESULT	ZONE 1 0-100μm	ZONE 2 100-200μm	ZONE 3 >200μm	ALL ZONES 0->200μm
TUMOUR 1	n	9.0	14.0	4.0	27.0
METAPHASE	χ	8.5556	11.5714	6.2500	9.7778
TO MID-	s	9.2616	5.2288	9.9791	7.4644
ANAPHASE	t	2.7713	8.2804	1.2526	6.8065
TUMOUR 1	n	15.0	2.0	4.0	21.0
MID-	χ	6.3333	-17.5000	-3.0000	2.2857
ANAPHASE TO	s	9.6634	10.6066	7.4386	11.6754
TELOPHASE	t	2.5383	2.3333	0.8066	0.8971
TUMOUR 2	n	20.0	11.0	NT	31.0
METAPHASE	χ	17.5500	10.2727		14.9677
TO MID-	s	9.9550	13.0391		11.4876
ANAPHASE	t	7.8840	2.6129		7.2545
TUMOUR 2	n	15.0	13.0	10.0	38.0
MID-	χ	-0.6000	-2.1538	-1.8000	-1.4474
ANAPHASE TO	s	8.3135	10.9761	9.9197	9.4689
TELOPHASE	t	0.2795	0.7075	0.5738	0.9423

KEY: n = *number of displacements;*

χ = *arithmetic mean of vector coordinates in millimeters*

s = *standard deviation of sample*

t = *t value from student's t-test*; NT= *no data to test*

TABLE 7.8.ii STATISTICAL RESULTS OF MEAN X DISPLACEMENTS IN NORMAL CULTURES

SAMPLE POPULATION	RESULT	ZONE 1 0-100μm	ZONE 2 100-200μm	ZONE 3 >200μm	ALL ZONES 0- >200μm
NORMAL 1 METAPHASE TO MID- ANAPHASE	n	6.0	8.0	NT	14.0
	χ	5.8333	3.3750		4.4286
	s	3.5449	3.4615		3.5887
	t	4.0308	2.7577		4.6174
NORMAL 1 MID- ANAPHASE TO TELOPHASE	n	4.0	NT	NT	NT
	χ	3.2500			
	s	5.5000			
	t	1.1818			
CONTROL NO MITOSIS	n	19.0	17.0	NT	36
	χ	2.3158	-2.3529		0.1111
	s	11.3727	11.6294		11.5728
	t	0.8876	0.8342		0.0576

KEY: n = *number of displacements;*

χ = *arithmetic mean of vector coordinates in millimeters*

s = *standard deviation of sample*

t = *t value from student's t-test;*

NT= *no data to test*

TABLE 7.8.iii STATISTICAL RESULTS OF MEAN Y DISPLACEMENTS IN TUMOUR CULTURES

SAMPLE POPULATION	RESULT	ZONE 1 0-100μm	ZONE 2 100-200μm	ZONE 3 >200μm	ALL ZONES 0- >200μm
TUMOUR 1	n	9.0	14.0	4.0	27.0
METAPHASE	χ	-1.1111	-2.4286	0.2500	-1.5926
TO MID-	s	5.6224	8.5008	8.9954	7.4949
ANAPHASE	t	0.5929	1.0690	0.0556	1.1041
TUMOUR 1	n	15.0	2.0	4.0	21.0
MID-	χ	0.6000	3.0000	6.2500	1.9048
ANAPHASE TO	s	7.8358	16.9706	8.6168	8.5843
TELOPHASE	t	0.2965	0.2500	1.4507	1.0168
TUMOUR 2	n	20.0	11.0	NT	31
METAPHASE	χ	-2.1500	3.0000		-0.3226
TO MID-	s	10.2970	17.0939		13.0700
ANAPHASE	t	0.9338	0.5821		0.1374
TUMOUR 2	n	15.0	13.0	10.0	38
MID-	χ	1.2667	-2.0000	6.9000	1.6316
ANAPHASE TO	s	11.7380	13.0320	10.9286	12.1842
TELOPHASE	t	0.4180	0.5533	1.9966	0.8255

KEY:

n = *number of displacements; NT= no data to test*

χ = *arithmetic mean of vector coordinates in millimeters*

s = *standard deviation of sample;*

t = *t value from student's t-test*

TABLE 7.8.iv STATISTICAL RESULTS OF MEAN Y DISPLACEMENTS IN NORMAL CULTURES

SAMPLE POPULATION	RESULTS	ZONE 1 0-100μm	ZONE 2 100-200μm	ZONE 3 >200 μm	All ZONES 0- >200μm
NORMAL 1 METAPHASE TO MID- ANAPHASE	n	6.0	8.0	NT	14.0
	χ	-7.1667	1.6250		-2.1429
	s	0.9832	5.0125		5.8554
	t	17.8548	0.9169		1.3693
NORMAL 1 MID- ANAPHASE TO TELOPHASE	n	4.0	NT	NT	NT
	χ	-1.0000			
	s	5.3541			
	t	0.3735			
CONTROL NO MITOSIS	n	19.0	17.0	NT	36.0
	χ	-3.8421	0.4118		-1.8333
	s	10.8999	6.0936		9.0947
	t	1.5365	0.2786		1.2095

KEY:

n = *number of displacements;*

χ = *arithmetic mean of vector coordinates in millimeters*

s = *standard deviation of sample*

t = *t value from student's t-test;*

NT= *no data to test*

TABLE 7.9 i) SUMMARY OF VECTOR ANALYSIS FOR ALL X COORDINATES

CULTURE	MITOSIS	NUMBER OF MITOSES	NUMBER OF LYMPHOCYTES	TYPE OF MOTION ZONE 1 0-100	ZONE 2 100-200	ZONE 3 >200	TOTAL 0->200
TUMOUR 1 MET-MID-ANAPHASE	10	4	t	t	r	t
TUMOUR 1 MID-ANAPHASE TO TELOPHASE	10	4	t	r	r	r
TUMOUR 2 MET-MID-ANAPHASE	5	20	t	t	NT	t
TUMOUR 2 MID-ANAPHASE TO TELOPHASE	1	8	r	r	r	r
NORMAL 1 MET-MID-ANAPHASE	3	1	t	t	NT	t
NORMAL 1 MID-ANAPHASE TO TELOPHASE	3	1	r	NT	NT	r
NORMAL 2 NONE	0	7	r	r	NT	r

Key: r = *random movement;* t = *taxis;* NT = *no data to test*

TABLE 7.9 ii) SUMMARY OF VECTOR ANALYSIS OF Y COORDINATES

CULTURE MITOSIS	NUMBER OF MITOSES	NUMBER OF LYMPHOCYTES	TYPE OF MOTION ZONE 1	ZONE 2	ZONE 3	ALL ZONES
TUMOUR 1 MET-MID-ANAPHASE	10	4	r	r	r	r
TUMOUR 1 MID-ANAPHASE TO TELOPHASE	10	4	r	r	r	r
TUMOUR 2 MET-MID-ANAPHASE	5	20	r	r	NT	r
TUMOUR 2 MID-ANAPHASE TO TELOPHASE	1	8	r	r	r	r
NORMAL 1 MET-MID-ANAPHASE	3	1	t	r	NT	t
NORMAL 1 MID-ANAPHASE TO TELOPHASE	3	1	r	NT	NT	r
NORMAL 2 NONE	0	7	r	r	NT	r

Key: r = *random motion;* t = *taxis;* NT = *no data to test*

DISCUSSION

The importance of comparing locomotion in separate zones was demonstrated in Tables 7.9 i) and ii). At distances greater than 200 μm from the mitosis the results were random although for the same lymphocytes when closer to the mitosis demonstrated taxis. The obvious reason for a random response of lymphocytes in the far zone is that whatever attractant is produced by mitotic cells becomes too weak to affect locomotion at 200 μm. However it must be noted that the sample number tended to be smaller because part of the zone was not visible on the screen and also off-screen mitoses may have occurred.

The results suggest that a lymphocyte attractant is produced by mitotic cells in monolayer cultures of tumour and normal laryngeal cultures, which diffuses into the medium, setting up a gradient, surrounding the mitotic cell. This is best seen from the data of tumour 2 (Table 7.9 i & ii). This culture had abundant lymphocytes and was suitable for testing the effective range of the attractant produced at mitosis, since lymphocytes were distributed randomly within 360 degrees of a 200 μm zone. Thus vector analysis was able to test the effect of a single tumour mitosis on several lymphocytes. Pathways shown in Figure 7.2 demonstrate clearly that most lymphocytes are oriented towards the mitosis until mid-anaphase when there is a rapid change in orientation, which was statistically shown to be a change from a taxis to random locomotion. This effect was observed throughout a 360 degree area.

In this study, types of random locomotion such as kinesis and contact guidance have been excluded by vector analysis. I have selected mitoses, in which there is a close association between at least one wandering

lymphocyte and the mitosis under study, on the assumption that random locomotion such as orthokinesis would result in movement to a different area than the source of the stimulus. In a kinesis actual arrival of a lymphocyte at a mitotic cell is less likely to occur, since it is only in taxis that locomotion is persistently directed towards the source of the attractant. This was considered the best way to exclude any movement due to kinesis.

T lymphocyte chemotaxis to mitotic carcinoma cells may also occur *in vivo*. It is known that carcinomas are predominantly infiltrated by T lymphocytes at the tumour edges. The heavy accumulation of T lymphocytes to this region may be a chemotactic response to mitotic cells, since it is here that tumour cell division is most evident. Rowe *et al*, (1984) noticed that lymphocyte subsets in carcinoma of the breast do not reflect a simple recruitment from peripheral blood. They suggested two theories: a) lymphocytes may divide in the carcinoma or b) lymphocytes are selectively recruited. The findings in this chapter suggest that mitosis is important in the selective recruitment of lymphocytes to the area surrounding the tumour and perhaps other regions of laryngeal carcinoma. A similar attraction of lymphocytes to mitotic megaloblasts was demonstrated by Pulvertaft in 1959, but his work lacked any statistical analysis.

The alternative possibility that lymphocytes may stimulate carcinoma cell mitosis could explain the proliferation of carcinoma cells in the presence of lymphocytes at the edges of the tumour. However in this study I observed no indication that lymphocytes stimulated mitosis of carcinoma cells.

CHAPTER 8

CONCLUSIONS

CONCLUSIONS

Laryngeal carcinomas have been grown with considerable success in primary explant culture using basic growth medium supplemented with serum. In preparation for culture the tissue matrix was not disrupted, thus retaining many of the cell-cell interactions of the original tissue, such as those between stroma cells and tumour cells. The medium was not supplemented with additional growth factors or a feeder cell system. Growth of these carcinomas by highly disruptive methods of primary culture, without additional growth factors or feeder layers, have previously met with few successes. This being the case and furthermore as human head and neck carcinomas are the most difficult of all mammalian cells to cultivate, it is probably fair to attribute the success of the method reported in this thesis to the presence of original tissue matrix. The same method and medium was found to be suitable for the culture of normal laryngeal epithelium which then became available for direct comparisons.

This model presented here is a substantial advance in the study by tissue culture of differentiated carcinoma cells. Some but not all cell processes occurring *in vivo* are found in this *in vitro* model. Thus tumour cultures were differentiated on the basis of intermediate filaments being present and did not stratify like stratified squamous tissue *in vivo*.

No obvious differences were found between tumour and normal epithelial cultures. Several features of normal and tumour cultures resembled tumour histology, such as anisocytosis and mitoses.

A further finding resulting from this method was the presence of T-lymphocytes beneath cultures. Laryngeal carcinomas are not unique among carcinomas in bearing an infiltrating lymphoid and myeloid population. It is suggested that this model of tumour-lymphocyte interaction may be useful in the immunological evaluation of other carcinomas and other tumours. The lymphocytes found interacting with laryngeal carcinoma cultures were not observed to lyse carcinoma cells, although some were identified as suppressor/cytotoxic T cells. An absence of motile helper/inducer T cells among tumour cultures was also observed. This finding was unexpected since these cells are more often motile than any other lymphocyte. The role of locomotion in the priming of cytotoxic T cells by suppressor cells has possible importance but was not studied here. No macrophages were identified in the motile cell population. This may be because carcinoma cells produce an inhibitor of macrophage locomotion. Similarly it is a possibility that there is an inhibitor of helper/inducer T cells.

There is no doubt that motile lymphocytes were derived from the explant as either tumour-infiltrating lymphocytes or from tumour vasculature. Attraction of lymphocytes to mitotic tumour cells has been shown by using time-lapse video microscopy and confirmed to be a taxis by vector analysis. The same phenomenon occurred in control cultures. There is a strong indication that this influence could stimulate lymphocytes to migrate out of explants. Although, as yet unidentified as a single stimulus or multiple stimuli, lymphocyte migration from the explant was linked specifically to **proliferating** tumour and normal monolayers. Failure to stimulate lymphocyte migration could be seen in bizarre cultures which proliferated for very short periods and were rarely found to have motile lymphocytes. The ability of a live culture to exert a chemical influence on lymphocytes would be appreciably greater than a culture which is in

stationary growth phase or which is non-viable. It was evident that there was a chemical stimulus, rather than an electrical stimulus, involved in the attraction of lymphocytes to mitotic cells, since the phenomenon was never observed if diffusion currents were present. This must have dissipated any chemotactic factors but was unlikely to reduce an electrical phenomenon. All sightings of lymphocyte-mitotic cell interactions took place while the monolayer was covered by a top coverslip, thus preventing diffusion of secreted chemotactic factor(s). As the culture proliferated the medium bathing the culture and explant would comprise an array of pharmacological metabolites including chemotactic factors, which would stimulate lymphocyte locomotion. This would explain the random locomotion observed in regions of culture, which were lacking in mitotic cells.

Lymphocytes migrate predominantly beneath laryngeal cultures, only rarely moving on the apical cell surface. Many lymphocytes were resistant to immuno-staining, in a random fashion. This was speculated to be caused by the irregular distribution of intermediate filaments within tumour and normal epithelial cells and random areas of culture which were more than one cell in thickness.

Lymphocyte motility was found to resemble closely that of splenic lymphocytes and peripheral blood lymphocytes stimulated *in vitro* and moving in collagen gels. This confirms that this model complements those using collagen gel for studying lymphocyte locomotion as locomotion does not appear different on cultures. However, the use of a viable tumour substratum for investigating tumour-infiltrating lymphocytes may prove more useful in functional studies of these lymphocytes.

Lymphocytes in carcinomas are an intriguing presence; are they friend or foe? The interaction of lymphocytes with carcinoma cells remains an unexplained activity. This thesis has sought to investigate this phenomenon and to suggest further work which may aid in a better understanding and may perhaps find application in *in vitro* studies related to human immunotherapy of carcinomas.

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APPENDICES

APPENDIX 1 **REAGENTS**

1. SUPPLIERS OF REAGENTS

acetone	BDH Ltd
acid fuchsin	BDH Ltd
alkaline phosphatase	Sigma Chemical Co Ltd
2-amino-2-methyl-1,3-propandiol	Aldrich Chemical Co Ltd
aniline blue	BDH Ltd
Araldite MY753 resin	Polaron Ltd
benzyl dimethylamine	Polaron Ltd
calcium chloride	BDH Ltd
collagen (type I)	Sigma Chemical Co Ltd
3.3 diaminobenzidine tetrachloride	Sigma Chemical Co Ltd
dibutyl phthalate	Polaron Ltd
dihydrogen orthophosphate	BDH Ltd
disodium hydrogen phosphate	BDH Ltd
dodecenyl succinic anhydride	Polaron Ltd
N,N-dimethylformamide	Sigma Chemical Co Ltd
Dulbecco's Minimal Essential Medium	Flow Labs; Inc
fast blue (compound 20)	Raymond A Lamb
fast red TR salt	Sigma Chemical Co Ltd
foetal calf serum	SeraLab Ltd
	Imperial Labs Inc
L-glutamine	Flow Labs; Inc
glutaraldehyde	Polaron Ltd
levamisole hydrochloride	Sigma Chemical Co Ltd
naphthol AS-BI phosphate	Sigma Chemical Co Ltd
naphthol AS-MX phosphate sodium salt	Sigma Chemical Co Ltd

new fuchsin	Sigma Chemical Co Ltd
orange G	BDH Ltd
paraformaldehyde	BDH Ltd
penicillin/streptomycin	Flow Labs; Inc
phosphate buffered saline solution	Flow Labs; Inc
phosphotungstic acid	BDH Ltd
potassium chloride	BDH Ltd
propylene oxide	BDH Ltd
sodium cacodylate	Polaron Ltd
sodium chloride	BDH Ltd
sodium hydroxide	BDH Ltd
sodium nitrite	Sigma Chemical Co Ltd
tris (hydroxymethyl methylamine) (Analar grade)	BDH Ltd

1.1 MONOCLONAL ANTIBODIES & REAGENTS FOR IMMUNOCYTOCHEMICAL STAINING

UCHT1, UCHT4, HML-1, 4KB128, IB5, Leu 3M, PKK 1, PKE, RGE 53 and RKSE 60 were kindly donated by the departments of Histology and Haematology at University College and Middlesex Medical Schools, London.

NKH-1	Becton Dickinson Ltd; California
TCRδ-1	T Cell Sciences Inc. Massachussets
Anti-CD7	Dako Ltd; High Wycombe, Bucks.
Leu 3a	Dako Ltd.
Cam 5.2	Becton Dickinson Ltd.
rabbit serum	Becton Dickinson Ltd.
vimentin	Labsystems

rabbit anti-mouse immunoglobulins (RAM)	Dako Ltd
peroxidase conjugated rabbit anti-mouse immunoglobulins	Dako Ltd
APAAP complex	Dako Ltd

FITC conjugated goat anti-mouse immunoglobulins	Dako Ltd
Anti-alkaline phosphatase 6/7/6	Dako Ltd

1.2 SUPPLIERS OF NON-REAGENT CONSUMABLES

Petri dishes	Flow Labs Inc
flasks	Flow Labs Inc
coverslips	Flow Labs Inc
aluminium specimen stubs	Polaron Ltd
Apathy's mountant	Raymond A Lamb Ltd

1.3 PREPARATION OF REAGENTS

APAAP COMPLEX

alkaline phosphatase	200 mg
alkaline phosphatase 7/6/7 MoAb	20 ml

APAAP complex was prepared 1 day before use and incubated 18 h at 4°C. Alternatively a commercially available preparation of soluble complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase was purchased from Dakopatts Ltd.

ARALDITE RESIN MIXTURE

Araldite MY 753 resin	49 g
dodecenyl succinic anhydride	49 g
benzyldimethylamine	1.5 g
dibutyl phthalate	2.7 g

Araldite resin, dodecenyl succinic anhydride and dibutyl phthalate were mixed for 2 h. Benzyl dimethylamine was added and mixing continued for 1 h.

AYOUB-SCHLAR KERATIN STAIN

Aniline blue, orange G and phosphotungstic acid were dissolved in distilled water and filtered before use.

aniline blue	0.5 g
orange G	2 g
phosphotungstic acid	1 g
distilled water	100 ml

acid fuchsin solution was prepared and filtered before use

acid fuchsin	5 g
distilled water	100 ml

CACODYLATE BUFFER 0.2 M

sodium cacodylate	21.4 g
calcium chloride (dihydrous)	0.6 g
distilled water	300 ml

Sodium cacodylate and calcium chloride were dissolved in 300 ml distilled water. The pH was adjusted to 7.3 by adding 0.2 M hydrochloric acid and the volume adjusted to 500 ml with distilled water to produce a 0.2 M stock solution. A working concentration of 0.1 M was prepared in distilled water.

COMPLETE MEDIUM

fetal calf serum (tested free of mycoplasma)	70 ml
L-glutamine	10 ml
penicillin 100 IU/ml; streptomycin 100 µg/ml	5 ml
Dulbecco's Minimal Essential Medium	415 ml

All reagents were filter sterilised by passing through a 0.22 µm pore size cellulose acetate filter.

DIAMINOBENZIDINE (DAB)

diaminobenzidine		5 mg
DAB buffer		10 ml
(3% hydrogen peroxide		100 µl)

DAB was dissolved in DAB buffer and hydrogen peroxide added immediately before application to specimens.

DAB BUFFER pH 7.6

0.2 M tris (24.2 g/L)		6 ml
0.1 N hydrochloric acid	approximately	9.5 ml
distilled water		9.5 ml

FAST RED SUBSTRATE

naphthol AS-MX phosphate sodium salt	8 mg
dimethylformamide	200 µl
0.1 M tris buffer pH 8.2	9.8 ml
fast red TR salt	10 mg
1 M levamisole (24 mg/100 ml dH_2O)	10 µl

naphthol AS-MX phosphate sodium salt was dissolved in dimethylformamide. This was diluted to 10 ml with tris buffer and levamisole was added. Immediately before application to specimens, fast red TR salt was combined with other reagents and the complete substrate was filtered onto specimens.

NEW FUCHSIN/PROPANDIOL (AN ALTERNATIVE TO FAST RED SUBSTRATE)

SOLUTION 1

0.2 M 2-amino-2-methyl-1,3-propandiol	2.5 ml
0.05 M tris buffer pH 9.7	7 ml
sodium chloride	85.7 g
levamisole	4 mg

Propandiol buffer was mixed with tris buffer and sodium chloride. Levamisole was added.

SOLUTION 2

naphthol AS-BI phosphate	5 mg
N,N-dimethylformamide	60 µl

Naphthol AS-BI phosphate was dissolved in dimethylformamide.

SOLUTION 3

5% new fuchsin	20 µl
4% sodium nitrite (freshly prepared)	50 µl

The new fuchsin was mixed with sodium nitrite and incubated for 60 seconds while agitating. Solutions 1 and 2 were mixed and solution 3 was added. The pH was adjusted to 8.7 by adding HCL. After mixing well, the complete substrate was immediately filtered onto specimens.

FAST BLUE SUBSTRATE

naphthol AS-MX phosphate alkali solution	0.5 ml
dimethylformamide	100 µl
fast blue compound 20	10 mg
1mM levamisole hydrochloride	200 µl

Naphthol AS-MX phosphate alkali solution was mixed with dimethylformamide. The remaining reagents were added.

GLUTARALDEHYDE 3%

glutaraldehyde (30%)	10 ml
cacodylate buffer (0.2 M)	50 ml
distilled water	40 ml

Glutaraldehyde was added to 40 ml distilled water and made up to 100 ml with cacodylate buffer.

OSMIUM TETROXIDE 1%

osmium tetroxide 2 x 1g vials	2 ml
distilled water	98 ml

Osmium tetroxide was added to distilled water and kept in a dark bottle at 2% stock concentration. A 1% working concentration was prepared in 0.1 M cacodylate buffer.

PARAFORMALDEHYDE

paraformaldehyde	1.0 g
phosphate buffered saline solution (PBS)	100 ml

Paraformaldehyde was dissolved in PBS by adding 1M sodium hydroxide until the solution became clear. The pH was adjusted to 7.4 and the solution filtered before use.

PHOSPHATE BUFFERED SALINE SOLUTION

disodium hydrogen phosphate	1.4326 g
potassium dihydrogen orthophosphate	0.1361 g
sodium chloride	4.003 g
potassium chloride	0.1118 g
distilled water	500 ml

These reagents were dissolved in distilled water, the pH was adjusted to 7.4 and the final volume made up to 500 ml. The preservative sodium azide (0.1%) may be added.

TRIS BUFFERED SALINE SOLUTION

sodium chloride	32 g

tris (hydroxymethyl methylamine)	2.42 g
1N hydrochloric acid	17.6 ml*
distilled water	4 L

* pH was adjusted to 7.6 after combining all other reagents.

APPENDIX 2 ADDITIONAL TABLES OF RESULTS

TABLE 1 PATIENT DATA AND TUMOUR CLASSIFICATION

PATIENT	SEX	AGE	DIFFERENTIATION	SIZE/VERT. EXTENSION
1	F	62	well	
2	M	62	poor	3.5 cm
3	M	65	well	4.5 cm
4	M	46	?	2.0 cm
5	M	66	well	6.0 cm
6	M	59	poor	
7	M	45	?	
8	M	65	well	1.5 cm
9	M	34	well	
10	M	45	moderate to well	
11	F	67	?	
12	M	50	moderate	
13	F	73	moderate to poor	
14	M	43	well	
15	F	61	?	
16	M	74	well	
17	M	63	poor	4.5 cm
18	M	57	?	
19	M	51	moderate to poor	2.5 cm
20	M	72	?	
21	M	56	?	
22	M	63	well	1.5 cm
23	M	65	poor	3.0 cm
24	M	45	well	2.0 cm
25	M	51	?	
26	M	62	well	
27	F	60	poor	4.0 cm
28	M	62	poor	
29	M	66	well	
30	M	53	?	2.5 cm
31	M	52	?	
32	M	48	poor	
33	M	59	?	
34	M	51	poor	1.0 cm
35	M	65	poor	
36	M	71	poor	
37	M	59	poor	
38	F	39	?	
39	M	57	?	
40	F	70	?	
41	M	74	well	2.3 cm
42	M	43	well	2.0 cm
43	M	40	poor	4.0 cm
44	M	57	moderate	2.5 cm
45	M	51	?	
46	F	61	moderate to poor	9.0 cm
47	M	56	well	1.5 cm
48	F	22	well	
49	F	57	?	
50	M	62	?	
51	M	62	moderate	
52	M	47	moderate	4.0 cm
53	M	63	moderate	

54	M	71	?	
55	M	49	moderate to poor	3.0 cm
56	M	59	well	3.0 cm
57	M	56	well	
58	M	72	well	
59	M	54	well	
60	M	84	well	2.0 cm
61	M	73	well	
62	M	38	moderate	
63	M	60	?	
64	M	39	moderate to well	
65	F	63	moderate to well	
66	M	53	well	
67	M	72	well	
68	M	55	well	
69	M	48	well	
70	M	57	poor	2.0 cm
71	M	66	moderate	4.0 cm
72	M	38	?	
73	M	58	moderate to well	3.0 cm
74	F	70	well	
75	M	72	poor	
76	M	50	?	
77	M	68	?	
78	M	59	?	
79	F	52	?	
80	M	34	well	
81	F	54	well	3.5 cm
82	F	60	well	
83	M	67	poor	
84	M	59	well	
85	F	79	well	
86	F	77	?	
87	M	56	?	
88	M	65	poor	
89	F	62	moderate	
90	M	61	?	
91	F	57	well	
92	M	50	well	
93	M	60	moderate	2.0 cm
94	F	34	well	2.5
95	M	52	well	4.0 cm
96	M	50	moderate to well	1.5 cm
97	M	56	moderate to well	4.0 cm
98	F	67	moderate	5 cm
99	F	48	poor	4 cm
100	M	51	well	
101	F	68	?	
102	M	72	?	
103	F	59	moderate	
104	M	58	well	
105	F	53	moderate	5.5 cm
106	M	50	?	
107	M	45	?	

KEY: M= male; F= female; ?= information unavailable because histological report incomplete.

TABLE 2 TYPE OF OPERATION AND RATIO OF MALE TO FEMALE PATIENTS

	MALE TO FEMALE RATIO
LARYNGECTOMIES:	66:12
PULL-UPS:	16:13

TABLE 3 AGE DISTRIBUTION

AGE GROUP	MALE PATIENTS	FEMALE PATIENTS
20-29	0	1
30-39	5	2
40-49	12	1
50-59	33	6
60-69	21	10
70-79	10	5
80-89	1	0

TABLE 4 SUCCESSFUL AND UNSUCCESSFUL ATTEMPTS TO CULTURE TISSUE.

KEY: + = growth; - = no growth; c = contaminated; NS = not sampled

PATIENT	NORMAL	TUMOUR	PATIENT	NORMAL	TUMOUR
1	+	+	54	+	-
2	+	+	55	+	+
3	+	+	56	+	+
4	+	+	57	+	-
5	+	-	58	c	c
6	c	c	59	+	+
7	-	-	60	-	+
8	+	+	61	+	-
9	+	-	62	+	c
10	NS	NS	63	+	-
11	+	-	64	+	+
12	+	+	65	c	c
13	+	+	66	+	+
14	+	-	67	+	+
15	+	+	68	c	c
16	+	+	69	+	+
17	+	+	70	+	-
18	-	-	71	+	+
19	+	+	72	+	+
20	+	c	73	-	+
21	+	-	74	+	-
22	+	-	75	NS	-
23	+	-	76	+	+
24	+	c	77	+	+
25	-	-	78	+	+
26	-	-	79	+	+
27	+	+	80	+	+
28	+	+	81	+	+
29	+	+	82	-	-
30	-	+	83	+	-
31	-	-	84	+	-
32	+	+	85	-	-
33	c	c	86	+	-
34	+	+	87	-	c
35	+	+	88	+	NS
36	c	c	89	+	+
37	-	-	90	c	c
38	-	-	91	-	+
39	c	c	92	-	-
40	+	c	93	+	c
41	+	+	94	+	-
42	+	+	95	+	+
43	+	-	96	-	+
44	+	+	97	+	+
45	+	+	98	+	-
46	+	+	99	+	-
47	+	+	100	-	-
48	-	+	101	+	c
49	c	c	102	+	c
50	-	-	103	+	+
51	+	c	104	-	-
52	+	+	105	+	+
53	+	+	106	c	c
			107	c	c

TABLE 5 SEM STUDY.

*KEY: + = viewed by SEM; * = known to have motile lymphocytes*

PATIENT	NORMAL	TUMOUR
1	+	+*
2		+*
3	+	+*
4		+*
9	+	
13	+	+*
14	+*	
17	+*	+*
20	+	
28	+	+*
30		+*
32	+	
34	+	+
35	+	+*
41	+	+
45	+*	+
53	+	+
55	+*	+*
56	+*	+*
60	+	+
62	+	
78	+*	
80	+	
81	+	+
95	+*	+
96		+*
97	+*	+*
102	+*	

TABLE 6 CULTURES TAPED TO DETECT MOTILITY

KEY: + = motility; - = no motility; NT = not tested; LM = lymphocyte attracted to mitotic cell.

PATIENT	NORMAL	TUMOUR
1	NT	+
2	+	+
3	NT	+LM
4	NT	+ (and - if bizarre)
5	+LM	NT
8	NT	+
12	+	+
13	NT	+
14	+	NT
15	+	+
16	NT	+
17	+	+
19	NT	- (bizarre)
21	+	NT
24	-	NT
27	+	+
28	NT	+
30	NT	+LM
32	-(bizarre)	+
34	NT	-(bizarre)
41	-	NT
42	+	+LM
43	+LM	NT
45	+	NT
46	+LM	NT
53	NT	-(bizarre)
55	+	+
56	+	+ (and - if bizarre)
57	-	NT
60	NT	-(slightly bizarre)
61	+	NT
62	-	NT
63	+	NT
66	-	-
67	+	+
69	+	+LM
70	-	NT
72	NT	+
73	NT	-
76	NT	+
77	NT	-
78	+	+
79	+	+LM
80	NT	-
83	+	NT
84	+	NT
86	+	NT
94	-	NT
95	+LM	NT
96	NT	+
97	+LM	+
98	+	NT
99	+	NT

102	+	NT
103	+	+
105	+	+

TABLE 7 SUMMARY OF CHEMOTACTIC SIGHTINGS

KEY: + = chemotaxis; -(L) = lymphocytes motile but no chemotaxis; NT = not tested.

PATIENT	NORMAL	DXT	TUMOUR
5	+	+	NT
43	+	-	NT
46	+	+	NT
95	+	-	NT
97	+	-	-(L)
3	NT	-	+
27	-(L)	+	+
30	NT	-	+
42	-(L)	-	+
69	-(L)	-	+
79	-(L)	-	+

TABLE 8 GROWTH OF NORMAL MUCOSA AND TUMOUR FROM IRRADIATED LARYNXES

*KEY: + = growth; - = no growth; c = contamination; NS = not sampled; * =chemotherapy*

PATIENT	NORMAL	TUMOUR
5	+	-c
8	+	+
13	+	+
14	+	-
16	+	+
20	+	-c
24	-c	-c
27	+	+
28	+	+
33	-	-c
35	+	+
36	-c	-c
37	-	-
40	+	-c
41	+	+
46	+	+
48	-	+
52	+	+
54	+	-
57	+	NS
59	+	+
60	-	+
61	+	-
64	+	+
66*	+	+
67	+	+
68	-c	-c
71	+	+
72	+	+
74	+	-
76	+	+
83	+	-
84	+	-
88	+	NS
93	+	-c
98*	+	-
103	+	+

TABLE 9 SUMMARY OF LYMPHOCYTE PHENOTYPES FOR MOTILE CELLS

STAIN	MAB	RESULT	SPECIMEN
fast red	UCHT1	?	patient 79 tumour 1
		2+; 1-	patient 27 tumour 1
		3+; 0-	patient 79 normal 5
		0+; 1-	patient 83 normal 5
		0+; 1-	patient 83 normal 6
		0+; 2-	patient 106 normal 1
		0+; 15-	patient 79 tumour 4
DAB		1+; 0-	patient 5 normal 4
FITC		0+; 1-	patient 12 normal 3
		0+; 7-	patient 12 tumour 1
		0+; 2-	patient 12 tumour 4
fast red	Leu 3a	3+; 0-	patient 14 normal 5
		0+; 1-	patient 79 normal 3
		0+: 1-	patient 79 normal 1
		0+; 1-	patient 83 normal 3
		0+; 14-	patient 14 normal 2
		0+; 1-	patient 27 tumour 5
		0+; 1-	patient 83 normal 4
DAB		0+;>1-	patient 79 tumour 3
		0+; 2-	patient 79 tumour 2
fast blue		0+; 2-	patient 4 tumour 1
FITC		0+; 8-	patient 12 tumour 2
		0+; 12-	patient 12 normal 1
fast red	UCHT4	6+; 1-	patient 14 normal 7
		0+; 1-	patient 79 normal 2
		0+; 2-	patient 79 tumour 2
		0+; 1-	patient 83 normal 1
		0+; 3-	patient 103 tumour 2
		2+; 0-; 1 lost	patient 4 tumour 1
		0+; 2-	patient 103 normal 3
		1+; 1-	patient 103 normal 4
		0+; 6-	patient 14 normal 1
		0+; 1-	patient 27 tumour 3
		0+; 2-	patient 27 tumour 6
FITC		2+; 13-	patient 12 normal 2
DAB	NKH1	0+; 3-	patient 103 tumour 2
	HNK1		
		0+; 1-	patient 103 tumour 3
		0+; 1-	patient 103 normal 1
		0+; 2-	patient 103 normal 3
		0+; 2-	patient 103 normal 4
		0+; 15-	patient 79 tumour 4
DAB	HML1	1+; 0-	patient 98 normal 1
		1+; 0-	patient 98 normal 3
		0+; 1-	patient 98 normal 2
		0+; 1-	patient 83 normal 1
		0+; 1-	patient 83 normal 3
		0+; 1-	patient 83 normal 4
		0+; 1-	patient 83 normal 5
		0+; 1-	patient 83 normal 6
		0+; 1-	patient 5 normal 7

		0+; 1-	patient 5 normal 8
		0+; 1-;	patient 5 normal 1
fast blue		0+; 3-	patient 103 tumour 2
		0+; 1-	patient 103 tumour 3
		0+; 1-	patient 103 normal 1
		0+; 2-	patient 103 normal 3
		0+; 2-	patient 103 normal 4
	CD7	0+; 1-	patient 5 normal 7
		1+; 0-	patient 5 normal 8
		0+; 1-	patient 98 normal 1
		0+; 1-	patient 98 normal 2
		0+; 2-	patient 98 normal 3
fast red	TCRδ1	0+; 6-	patient 27 tumour 2
		0+; 4-	patient 14 normal 6
FITC		0+; 8-	patient 12 tumour 3
DAB	αIL-2	0+; 1-	patient 5 normal 3
DAB	Leu 3M	0+; 1-	patient 103 tumour 3
		0+; 1-	patient 103 normal 1
fast red	4KB128	0+; 4-	patient 14 normal 3
		0+; 2-	patient 27 tumour 4
	Ki67	0+; 1-	patient 14 normal 4
		0+; 2-	patient 27 normal 1
		0+; 1-	patient 27 normal 2

APPENDIX 3 METHODS

All reagents were the purest available. A list of reagents, suppliers and chemical or media preparation is presented in Appendix 1. Tissue culture reagents were prepared under aseptic conditions and stored at 4°C.

3.1 TRANSMISSION ELECTRON MICROSCOPY

Prefixation of specimens.

Biopsy portions taken from freshly excised tumour and true cord, measuring $2cm^3$ were pre-fixed in 3% (v/v) glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 for 1 h. Cultures of tumour and true cord were treated in the same way.

Processing technique for transmission electron microscopy.

Cultures were scraped from coverslips with a scalpel. Biopsy specimens were cut into 1 mm^3 fragments.

All steps were carried out at room temperature in a fume-extractor safety cabinet. Details of reagents and methods for preparation are given in Appendix 1.4.

Specimens were processed as outlined below:

a) Three washes in 0.1 M cacodylate buffer pH 7.4 for 15 mins.

b) Post-fixation in 1% (v/v) osmium tetroxide in 0.1 M cacodylate buffer pH 7.4 for 1h.

c) A single wash in 0.1 M cacodylate buffer pH 7.4 for 15 min.

d) Three washes in distilled water for 10 min.

e) Sequential dehydration in increasing ethanol concentrations: 30%, 50%, 70%, 85% and 95% (v/v) in distilled water.

f) Three further dehydration washes in absolute alcohol for 15 min.

g) Two washes in 100% propylene oxide for 15 min.

h) Gradual embedding in propylene oxide and Araldite resin ratios: 70%:30%, 50%:50% for 2 h; 30%:70% overnight while held in a rotating rack.

i) Embedding in 100% Araldite resin for a minimum of 24 h.

j) Final embedding in fresh resin in mould for a minimum of 24 h at 60°C.

k) Remove from mould and allow to cure for 1-2 days before cutting.

Section preparation and microscopy

Thin sections were cut on a Reichert-Jung Ultracut E microtome and stained with toluidine blue. After selecting a suitable site, ultrathin serial sections were cut and placed on fine grids. Sections were stained as follows:

a) Section was stained in 7% uranyl acetate for 25 min.

b) Section was washed in distilled water.

c) Section was stained in Reynold's lead citrate for 15 min.

d) Section was washed in distilled water and dried on filter paper.

Sections were viewed by a Jeol 100-S transmission electron microscope. Micrographs were produced from single plate film FP4 125 ASA.

3.1.1 SCANNING ELECTRON MICROSCOPY

Cultures were left adhered to coverslips.

Follow steps a-f of 'Processing technique for transmission electron microscopy'.

g) Cultures were critical-point dried (Polaron Equipment Ltd, Watford)

h) Coverslips were mounted on aluminium specimen mounts (Polaron Equipment Ltd) using adhesive discs (Agar Scientific Ltd).

i) Cultures were sputter-coated with a mixture of gold and palladium 60:40 in an argon gas atmosphere using a sputter-coater (Edwards S150B)

Specimens were viewed on a Jeol JSM-35C scanning microscope. Micrographs were taken using a Mamiya camera back and FP4 125 ASA, 60 mm 220 print film.

3.2 IMMUNOCYTOCHEMICAL STAINING

All cultures for PAP and APAAP methods were fixed in acetone at room temperature for 5 min. Sections of larynx were fixed for 30 min. Cultures for immunofluorescence were fixed in paraformaldehyde in PBS at pH 7.2-7.4 at room temperature for 5 min.

3.2.1 PRIMARY MONOCLONAL ANTIBODIES

CLUSTER OF DIFFERENTIATION AND ANTIBODY NAME	DILUTION PAP/APAAP	DILUTION IF	SPECIFICITY
CD3 UCHT1	1/50	1/50	Pan T cells
CD4 Leu3a	1/100	1/100	helper T cells macrophages
CD8 UCHT4	1/800	1/100	cytotoxic/suppressor T cells
CD7	1/25		T cells null cells
HML1	1/200		human IEL
NKH1	1/200		NK cells
TCRδ-1	1/50	1/50	double negative T

		cells:
		(CD4-/CD8-/CD3+)
CD25 (IL-2 receptor)	1/200	activated T cells
		IL-2 receptor-
		bearing T cells
		macrophages
αHLA-DR (IB5)	1/1	B cells
		activated T cells
		macrophages
CD22 (4KB128)	neat	B cells
P150.95	1/50	macrophages
Leu3M	1/100	macrophages

ANTI-KERATIN MONOCLONAL ANTIBODY	**DILUTION**	**SPECIFICITY**
PKK1	1/200	general for cytokeratins
PKE (polyclonal)	1/8	general for cytokeratins
RGE 53	1/5	cytokeratin 18
CAM 5.2	neat	low molecular weight keratins
		39, 43 & 50 kDa
RKSE 60	1/5	suprabasal epithelial cells
		and squamous cell carcinomas
Vimentin	1/5	mesenchymal cells 58 kDa

3.3 PAP METHOD: SINGLE MONOCLONAL ANTIBODY INDIRECT PEROXIDASE ANTI-PEROXIDASE TECHNIQUE.

Cultures were fixed in acetone at room temperature. For anti-cytokeratin staining, 30 mins and for all others 5 mins. Endogenous peroxidase was inhibited in cultures to be stained with anti-cytokeratin monoclonal antibodies, by incubating with 3% (v/v) hydrogen peroxidase for 10 mins. All procedures were carried out at room temperature in a humidified chamber. Reagents were prepared in tris buffered saline solution (TBS) pH 7.6. All suppliers and preparation details are given in Appendix 1.1 and Appendix 1.3, respectively. All primary monoclonal antibodies were raised in mouse.

a) After washing well in TBS, primary antibody was applied to cultures and control sections for 1h at concentrations given in Appendix 3.2.1. Rabbit serum diluted to 1/200 was used as a negative control for anti-cytokeratin stains. TBS was used as a control for all other stains.

b) Specimens were twice washed in TBS for 5 min.

c) Secondary antibody: peroxidase conjugated rabbit anti-mouse immunoglobulins (RAM-P) was applied for 30 min at 1/50 containing normal human serum at 1/25 to block non-specific tissue reaction.

d) Specimens were twice washed in TBS for 5 min.

e) An enhanced staining reaction was achieved by application of swine anti-rabbit immunoglobulin at 1/100 for 30 min.

f) Specimens were twice washed in TBS for 5 min.

g) The chromagen diaminobenzidine was applied for 10 min which produces a coarse brown/black precipitate when bound to the peroxidase anti-peroxidase product.

h) Specimens were washed in tap water, viewed microscopically and photomicrographs of positive cells were taken. Haematoxylin was used as counterstain and further photomicrographs were taken.

3.4 APAAP METHOD: SINGLE MONOCLONAL ANTIBODY ALKALINE PHOSPHATASE ANTI-ALKALINE PHOSPHATASE TECHNIQUE.

a) Primary monoclonal antibody was applied for 1h.

b) Specimens were washed twice in TBS.

c) Secondary antibody: rabbit anti-mouse immunoglobulin (RAM) at 1/50 with normal human serum at 1/25 were applied for 30 min.

d) APAAP complex was applied for 30 min.

e) Steps c & d were repeated for 15 min to produce a stronger staining product.

f) Early chromagen choice was fast red substrate, but was replaced by the new fuchsin/propandiol method (see Appendix 1.3) which produced a stronger staining product. This was applied until sufficient red colour was observed microscopically.

g) Specimens were washed in distilled water, viewed microscopically prior to counterstaining for positive cells and then counterstained with haematoxylin without dehydration in alcohol since both the chromagens are alcohol labile. For the same reason an aqueous mountant Apathy's mountant (Raymond A Lamb, London) was used.

3.5 SEQUENTIAL DOUBLE STAINING

A combination of stains may be applied in sequence to assay for two antigens.

3.5.1 SEQUENTIAL APAAP STAINING TECHNIQUE

Steps a-f of APAAP technique section above were followed.

g) A second primary antibody was applied for a minimum of 1h.

h) Specimens were washed twice in TBS for 5 min.

i) Secondary antibody: rabbit anti-mouse immunoglobulin (RAM) at 1/50 with normal human serum 1/25 were applied for 30 min.

j) Specimens were washed twice in TBS for 5 min.

k) APAAP complex was applied for 30 min.

l) Specimems were washed twice in TBS for 5 min.

m) The chromagen fast blue substrate was applied, while incubating at 37°C. Incubation time was assessed microscopically to prevent over-development of background non-specific blue colour rendering red cells black.

n) Specimens were washed in TBS and finally in distilled water.

o) Photomicrographs were taken before and after minimal haematoxylin counterstaining (avoiding alcohol dehydration or alcohol-based mountant).

3.5.2 SEQUENTIAL PAP AND APAAP STAINING TECHNIQUE

Steps a-g of PAP method above were followed.

h) A second primary antibody was applied overnight to cultures kept in a moist chamber at 4°C.

i) Specimens were washed twice in TBS for 5 min.

j) Secondary antibody: rabbit anti-mouse immunoglobulin (RAM) at 1/50 with normal human serum at 1/25 were applied for 30 min.

k) Specimens were washed twice in TBS for 5 min.

l) APAAP complex was applied for 30 min.

m) Steps j-l were repeated for 15 min.

n) Specimens were washed twice in TBS for 5 min.

o) The chromagen fast red substrate (later replaced by new fuchsin/propanediol) or fast blue substrate was applied as described in sections above.

p) Specimens were washed in TBS, followed by distilled water and mounted in Apathy's mountant.

3.6 IMMUNOFLUORESCENT STAINING

The following procedure was carried out on cultures on glass coverslips placed in a moist chamber.

a) Specimens were washed twice in phosphate buffered saline (PBS) at pH 7.4 containing 0.1% (w/v) sodium azide (to prevent capping) for 5 min.

b) Primary monoclonal antibody at concentration given in this Appendix was applied for 1.5 h.

c) Specimens were washed twice in PBS for 5 min.

d) Secondary antibody conjugated to fluorescent marker: goat anti-mouse-FITC at 1/20 was applied for 1h, keeping specimens in darkness.

e) Specimens were washed twice in PBS for 5 min.

f) Specimens were mounted in glycerol and viewed as soon as possible. In case specimens were required at a later time, it was necessary to seal the coverslip to the slide with nail varnish and store at 4°C. This reduced evaporation of water from mountant.

g) A phase-contrast microscope which also had fluorescent optics was needed to locate the site of motile cells

h) Results were recorded on transparencies (Fujichrome P1600 ASA).

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Video time-lapse microscopy of human laryngeal carcinomas *in vitro*

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Video time-lapse microscopy of human laryngeal carcinomas *in vitro*

Twelve human laryngeal carcinomas and 14 normal vocal cord epithelia were studied *in vitro* by the method of tissue culture incorporating video time-lapse microscopy. Different events were photographed by taking 'stills' from the television monitor during play-back. Outgrowths from both tumour and normal explants formed only as monolayers. Mitoses were mainly found to be localized approximately mid-way between explant and the edge of the outgrowth. Some cells were seen to become suddenly active producing oscillating blebs and sometimes they detached themselves from the monolayer culture and changed appearance. This was more prevalent in the tumour cultures. In both tumour and normal cell cultures lymphocytes were observed and their normal chemokinetic movement was changed into a rapid chemotactic attraction by the presence of a mitotic epithelial cell. These lymphocytes appear to represent T-suppressor cells. The present study has shown that primary tissue culture can be a valuable tool in the study of laryngeal carcinoma. The interaction between mitotic tumour cells and lymphocytes may represent a transformation of T-lymphocytes into LAK cells.

Keywords *larynx tissue culture time-lapse microscopy lymphocytes*

Cell lines of human laryngeal squamous cell carcinomas have been surprisingly difficult to establish and there are few laboratories claiming successful results.[1–4] Primary tissue culture does provide satisfactory results, however, and by incorporating time-lapse videophotography it is possible to study several aspects of human tumour cells. This paper briefly describes the method of establishing primary tissue culture of human laryngeal carcinomas and their videophotography and describes the findings using the latter technique.

Materials and methods

TISSUE CULTURE

Tissue from tumour and true vocal cord was dissected from fresh laryngectomy specimens under sterile conditions. The tissue was washed with 3 changes of DMEM containing penicillin 100 IU/ml

Part of this work was presented at the Otorhinolaryngolic Research Society Meeting, London, October 1985.

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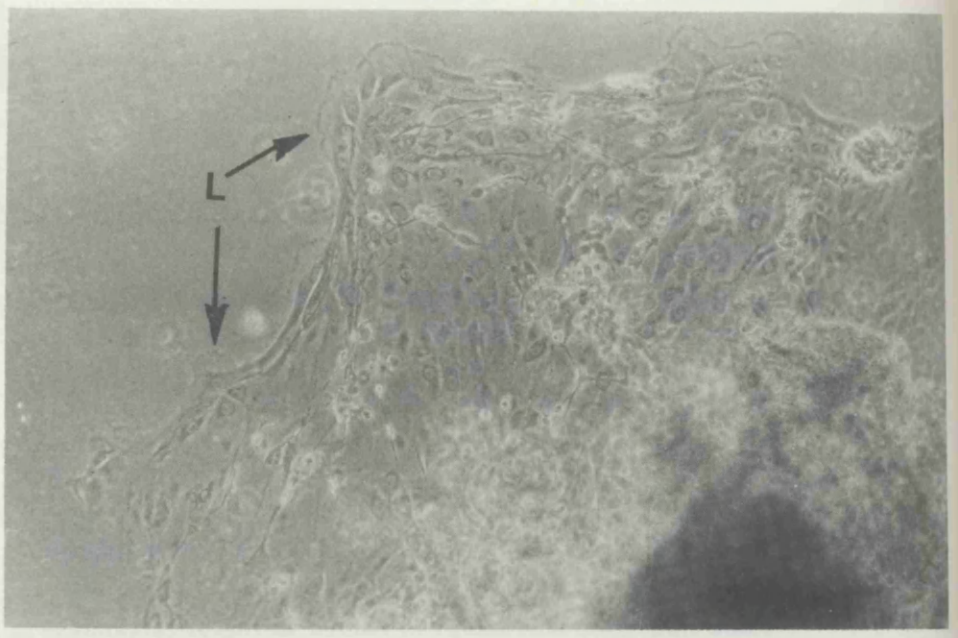


Figure 1. Outgrowth from a laryngeal squamous cell carcinoma where the leading lamellae (L) are seen at the edge. (× 25).

and streptomycin 100 μg/ml to reduce the level of microbiological contamination. The tissue was then cut into small pieces, approximately 2 mm,[3] which constituted the actual explants. These explants were sandwiched between sterile coverslips (which were sealed) in tissue culture Petri dishes.[5] Growth medium was then added and the cultures incubated in a water-saturated atmosphere of 5% CO_2 in air at 37°C for 7–10 days, during which time the cultures were left undisturbed. Growth was then checked every 2 days when the growth medium was also changed. Details of the tissue culture method and media constituents have recently been published elsewhere.[6] Twelve tumour and 14 normal vocal cord epithelium growths from 6 patients have so far been investigated by video time-lapse microscopy.

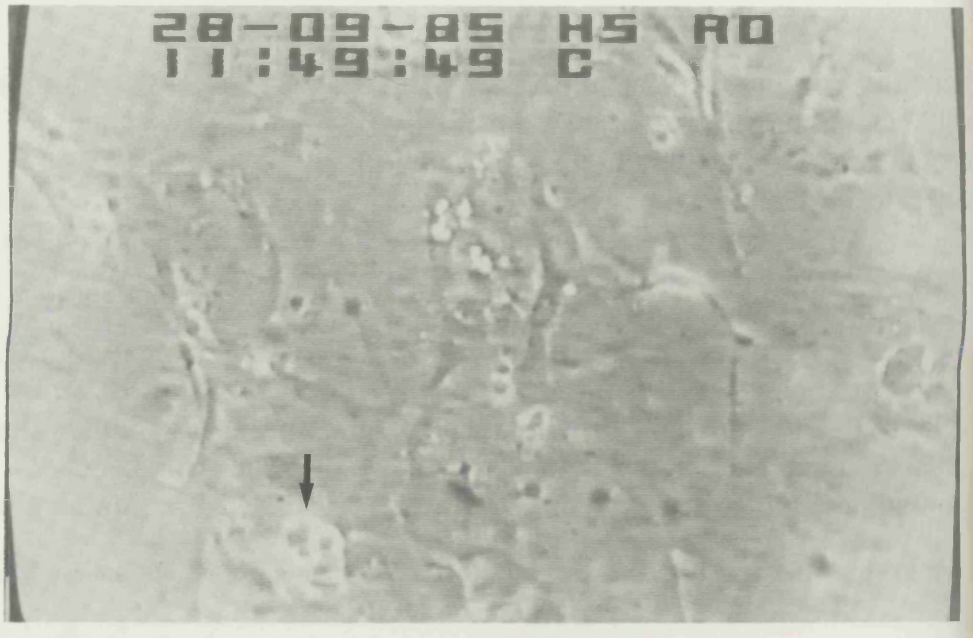


Figure 2. Photograph of tumour outgrowth taken directly from the television screen where an 'oscillating' cell is seen (arrow).

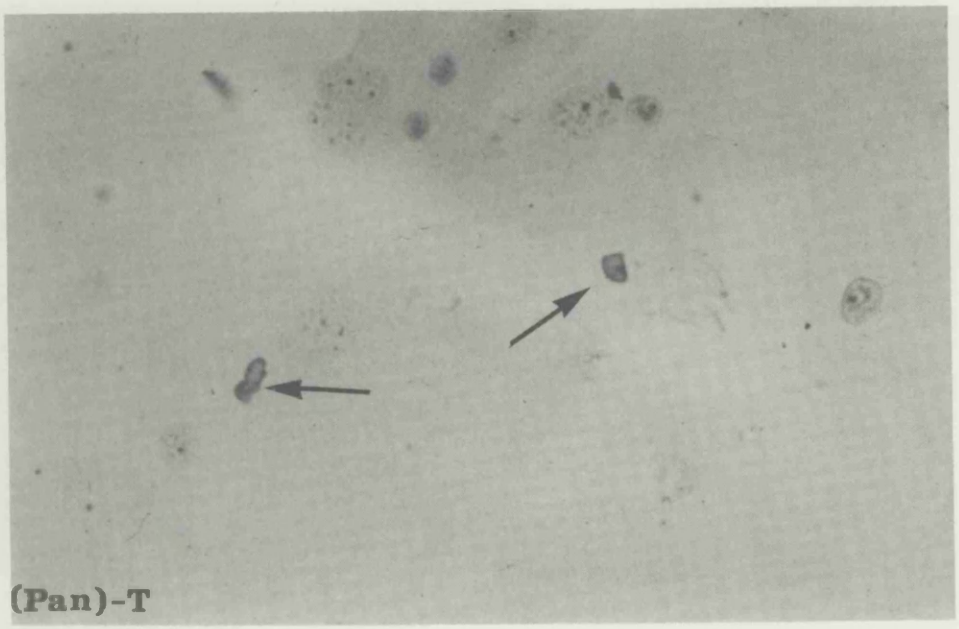


Figure 3. Tumour outgrowth with positive staining with T-cell antiserum. (PAP; × 320).

VIDEO TIME-LAPSE MICROSCOPY

To be able to film and record growing cultures over a longer period of time, the environment for the cultures under the inverted microscope must closely resemble that in the incubator. Therefore an incubator was attached to the microscope (Nikon Diaphot phase-contrast inverted microscope), and inside this incubator-cabinet the temperature was maintained at 37°C by a small heater. The actual Petri dish supporting the culture was placed inside a gassed flask. An Hitachi CCTV camera was attached to the microscope with a Panasonic time-lapse recorder. For routine time-lapse recording the 10x objective of the microscope was used. This arrangement made it possible to film and record pictures automatically every 2 s on a 180-min VHS videotape and to play these back at normal speed. This enabled long-term studies to be conducted. The recorder can be programmed to record on 3-h tape

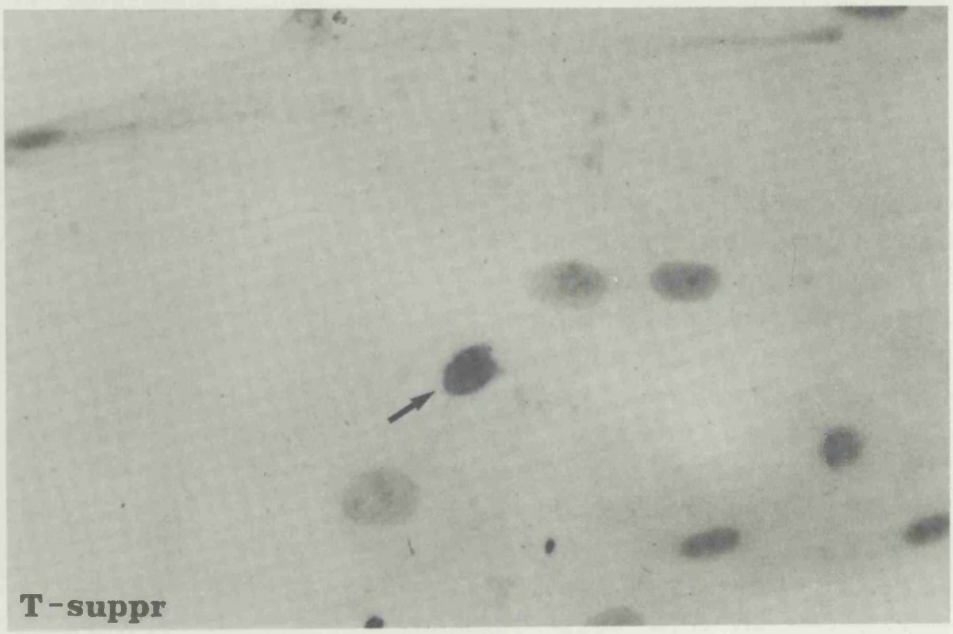
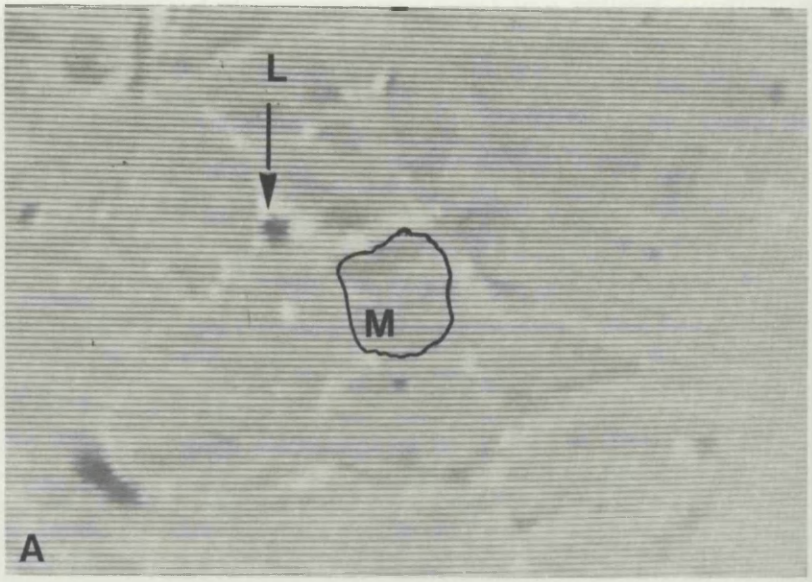


Figure 4. Positive staining with a T-suppressor antiserum. (PAP; × 500).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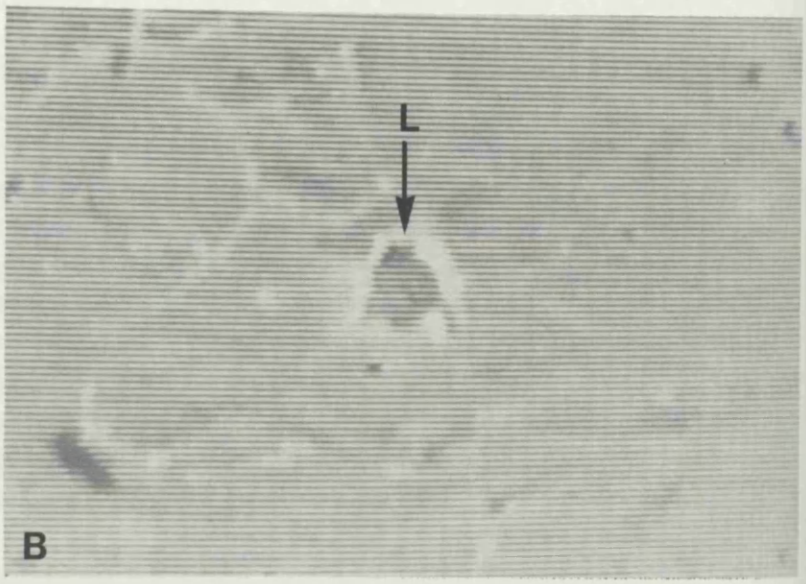
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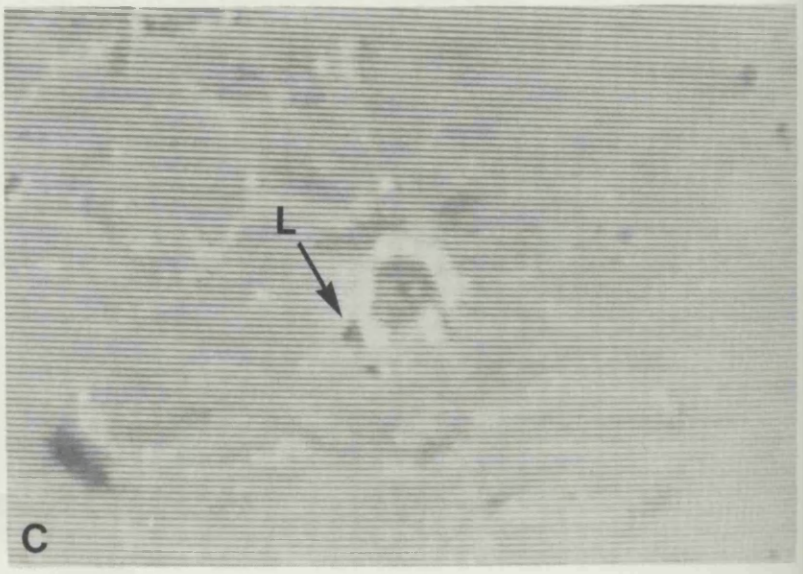
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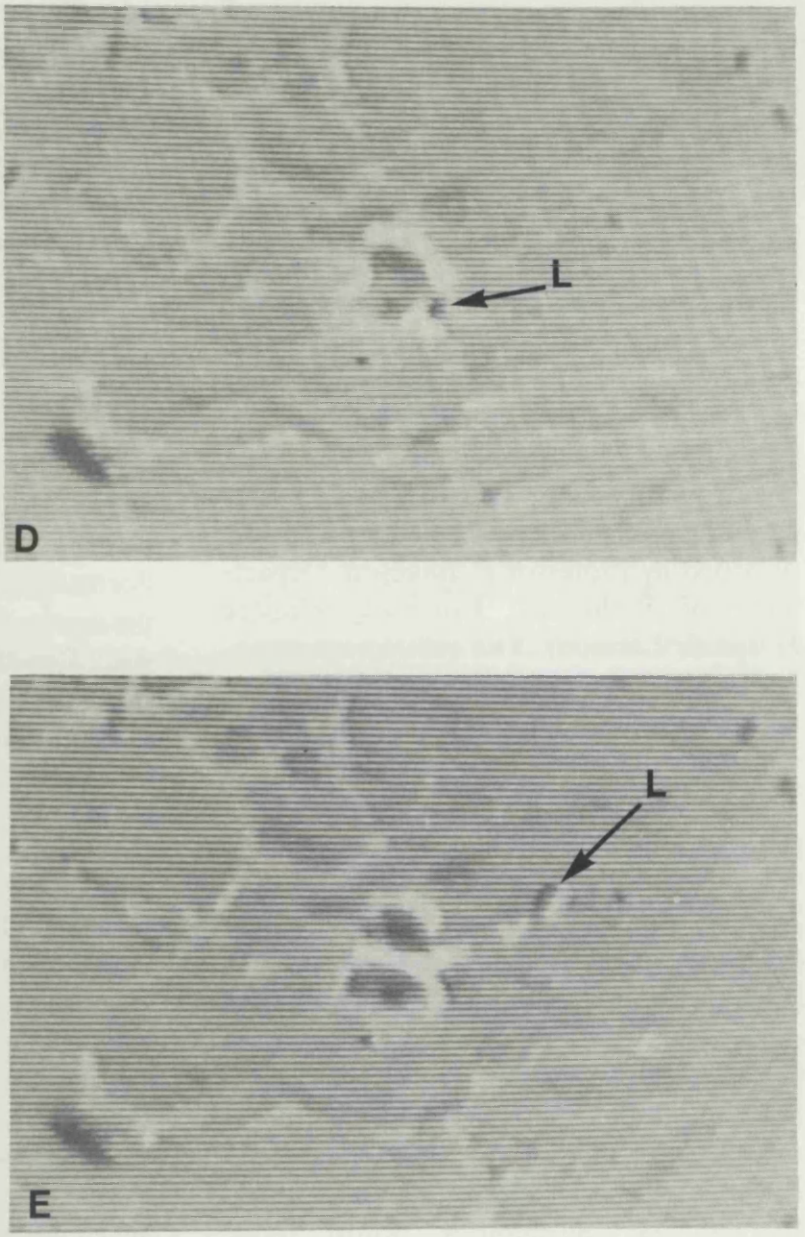

Figure 5. Outgrowth from true vocal cord. (**A**). In the centre there is an epithelial cell (M) entering mitosis and an approaching lymphocyte (L). The lymphocyte circumnavigates the cell in mitosis twice (**B–D**) and finally it is leaving the site of mitosis (**E**).

at suitably protracted film speeds, so that a 3-h film can record for any of the following time periods: 12, 24, 48, 72, 120, 144 and 240 h. The latter time was found to be the most useful. The tape can also be played back at double speed which results in 10 days growth being observed in the space of $1\frac{1}{2}$ h. Observations were noted and photographs taken of interesting sightings.

PHOTOGRAPHY

Still photographs of events are needed for comparison purposes. Microphotographs could be taken with the ordinary camera attached to the microscope. However, this would require constant attention which is virtually impossible. Sequences of events can be photographed as still pictures directly from the television monitor screen holding the tape on pause at each significant alteration of the picture. This requires shutter speeds below one-fifteenth second and the camera mounted on a tripod. The quality of the pictures is not as good as if taken with the microscope camera focused down the microscope tube,

but quite adequate to record sequences as 'stills'. The date and time is automatically displayed on the screen by the recording equipment.

IMMUNOCYTOCHEMISTRY

Immunochemical investigations were carried out on lymphocytes in tissue culture. The cultures were fixed in acetone for 30 min before applying monoclonal antibodies specific for T-lymphocytes (Pan-T), B-lymphocytes (Pan-B) and T-suppressor cells. (The antibodies were kindly provided by Professor P. Isaacson, Department of Pathology, University College Hospital, London). The indirect immunoperoxidase method was used to detect antigen-antibody binding.

Results

CELL MOVEMENT AND MITOSIS

Outgrowths of tumour and of normal explants formed only as monolayers. The cells from the tumour material were particularly closely associated with neighbouring cells and both were attached to the substrate in sheet form. Individual cells within a sheet often retained their characteristic morphology, whilst those positioned at the edge of the outgrowth developed leading lamellae (Figure 1). Mitotic activity resulted in pressure on adjacent cells in order to accommodate new cells. Mitosis was commonly restricted to the central portion of the outgrowth, which resulted in some cells being moved towards the leading edge and others towards the explant. Cells seen to be pushed away passively from the mitotic area constantly changed their form.

In some cultures cells were seen to become active, suddenly producing oscillating blebs caused by a thrashing movement from within the cytoplasm (Figure 2). Although such cells did not stray from the initial site they sometimes detached themselves from the monolayer culture and changed appearance, becoming more spherical. On a few occasions such 'free' spherical cells attached themselves to or near the edge of the growing area where they resumed their flat spreading appearance.

'LYMPHOCYTIC' ACTIVITY

In all cultures there were other cells of an entirely different morphology. Some had a chemokinetic movement whilst others were dormant. Some of these cells stained positively with Pan-T antiserum but not with Pan-B (Figure 3). Furthermore they were positive with antiserum to T-suppressor antiserum (Figure 4). In 7 out of the 12 tumour cultures and 8 of the 14 normal epithelial cultures these 'lymphocytes' appeared to be attracted to epithelial cells in mitosis, moving in from as far as 0.2 mm and changing their random chemokinetic movement to a rapid chemotactic one. The change in speed was measured and found to be from approximately 3 μm/min (chemokinetic) to approximately 7 μm/min (chemotactic). After approaching the epithelial cell undergoing mitosis, the lymphocytes circumnavigated it, and were then repelled or moved away when cleavage was completed (Figure 5).[7] After this event the 'lymphocyte' seemed to increase in size.

Discussion

Video time-lapse microscopy enables detailed studies of cell movement and interaction between epithelial and lymphocytic cells in tissue culture to be carried out. By photographing the videotape replay from the television screen it is possible to outline individual cells and to observe subtle differences in cell movement. Furthermore intracellular events can be studied with relative ease, and the

technique may be especially valuable in the study of endocytosis and lysosomal activity.[8] The present study has shown that tumour cultures like normal cultures grow only as monolayer, having leading lamellae on peripheral cells. Similar forces of contract interaction, therefore, most probably apply equally to both types of cultures.

In both tumour and normal cultures mitotic figures were more prevalent in areas towards the central part of the outgrowths. This localization of mitoses could be due to the surroundings being suitable for the expansion, i.e. optimal physical pressure from surrounding cells.

The observation specific to tumour cultures in which cells were seen to produce blebs and display rather violent movements cannot be explained, but similar actions have been reported in isolated normal epithelial cells.[9, 10]

All 'lymphocytes' appeared to be T-cells as identified by immunocytochemical staining. However, not all lymphocytes were motile, some appearing to remain dormant. For this reason it is important to compare the filmed culture with the same stained culture to identify these motile cells. After the change into a chemotactic movement and when the mitosis was completed the lymphocytic cell became somewhat larger. It may be possible that the lymphocyte was exposed to a lymphokine (Interleukin 2) by the mitotic cell and transformed into a Lymphokine-Activated Killer cell.[11]

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