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Lung stem cells

W.R. OTTO
Histopathology Unit, Imperial Cancer Research Fund, London, UK.

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Summary. The identity of putative stem cells in tracheobronchial, bronchiolar and alveolar compartments is reviewed. Their developmental appearance is described, as is the experimental evidence for their ability to repopulate areas of damage caused by noxious agents such as ozone, mineral fibres, chemicals and mechanical or enzymatic removal. Factors affecting pulmonary cell proliferation and differentiation are considered. The role played by these cells in carcinogenesis is also discussed, with consideration of the position of neuroendocrine cells in this process.

Keywords: stem cells, development, neuropeptides, mineral fibre, wound healing, carcinogenesis

Human lungs are remarkable organs. Through a 300 m^2 epithelial–gas interface they process 10 000 l of air per day (Hopkin & lane 1996), and during exercise 2.5 l of oxygen per minute is absorbed into the blood (Weibel & Taylor 1996). Under normal conditions, air is nonabrasive and so lung damage through breathing is minimal. However, if epithelial damage occurs, the tissue can respond by cell renewal. This may occur when small particles are inhaled, which may fail to be entrapped in the upper conducting airways (Lesur *et al*. 1992b). Several noxious gases (NO_2, O_3) (Evans *et al*. 1975, 1986; Tanswell *et al*. 1990; Dumler *et al*. 1994; Rajini & Witschi 1995), fibres (Woodworth *et al*. 1983; Sesko *et al*. 1990; Bonner *et al*. 1991; Marsh & Mossman 1991; Heintz *et al*. 1993; Janssen *et al*. 1994; Marsh *et al*. 1994) or chemicals (Roemer *et al*. 1993) may also result in cell death. Such damage needs repair, and lung epithelia have a selection of options to achieve this. Epithelial cells which retain a proliferative function are located in each histological area within the lung, and their positions and progeny are now quite well understood. In this article the evidence for proliferation that follows lung damge is reviewed, and related to the ability of those cells to act as stem cells and to repopulate all the histological cell types seen in the vicinity. The role of these cells in tumourigenesis is also considered.

Correspondence: Dr W.R. Otto, Histopathology Unit, Imperial Cancer Research Fund, 44, Lincoln's Inn Fields, London WC2 A 3PX, UK. Fax: +44 (0)171 269 3491, e-mail: w.otto@icrf.icnet. uk

Normal pulmonary stem cells

There are many prior articles on lung structure and kinetic organization to which the interested reader is directed (Wright & Alison 1984; Jeffery 1987; Keenan 1987; Plopper & Dungworth 1987; Creagh & Krausz 1992; Weibel & Taylor 1996). The histology of the distal lung shows a simple epithelium, with areas of pseudostratified cells in the proximal conducting airways (trachea and bronchi), a more cuboidal epithelium in the terminal conducting area (bronchioles), and with flattened squamous cells within the alveoli (Figure 1). Each compartment has cells capable of renewal and which may act as stem cells. Firstly, the 'basal' cell of the trachea and bronchi (but also possibly the mucous secretory cell; see below), secondly the Clara cell of the bronchioles and thirdly the type II pneumocyte of the alveoli. Much early evidence was obtained from animal work where pulse labelling of cohorts of cells with tritiated thymidine (^{3}H-TdR) or bromodeoxyuridine (BUdR) was undertaken, and over a time course a series of epithelial

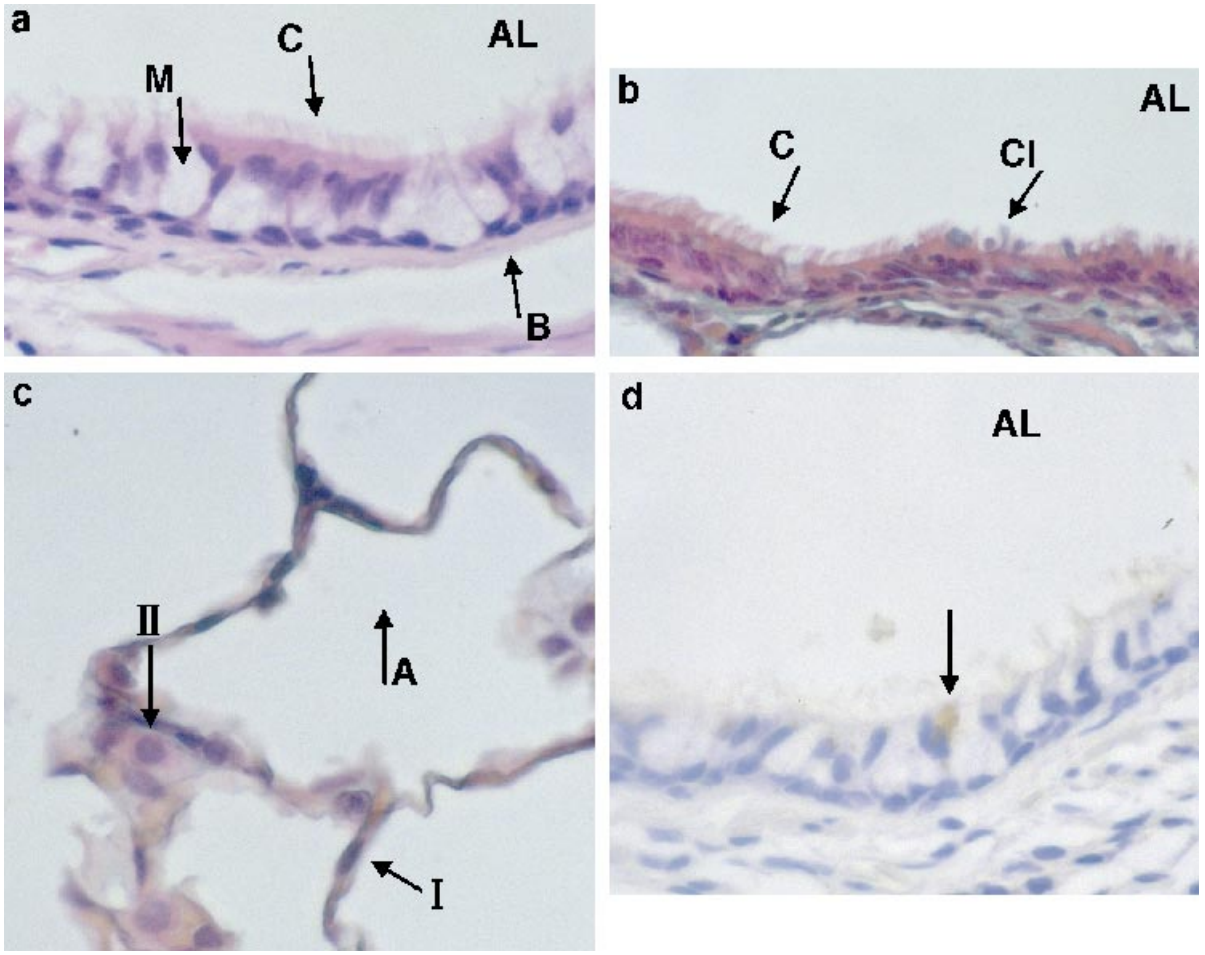

Figure 1. (a) Adult human tertiary bronchus with basal (B), mucous (M) and ciliated (C) cells. AL, Airway lumen. Original magnification 400X. Haematoxylin and Eosin. (b) Adult human bronchiole showing Clara cell (Cl) and Ciliated cell (C); AL, Airway lumen. Original maginfication 400x. Papanicolaou stain. (c) Adult human alveolus (A) showing flattened Type I Pneumocyte (arrow I) and Type II Pneumocyte (arrow II) with cytoplasmic vesicles containing surfactant. Original magnification 400x. Papanicolaou stain. (d) Adult human tertiary bronchus showing neuroendocrine cell (arrow), containing darkly staining cytoplasmic granules. Original magnification 400X. Immunocytochemical detection of Chromogranin A, revealed with DAB

labelling index (LI) measurements recorded (Wright & Alison 1984). Recently, experiments using rat, hamster, rabbit and other mammalian *in vivo* and *in vitro* models have broadly supported the earlier data. One aspect to bear in mind in the discussions below is whether the stem cell biology of tracheal 'basal' cells may be comparable to the basal cells of the bladder and cornea (Otani 1987) or to crypts of the gastrointestinal tract, or the so-called epidermal proliferative unit of mouse skin, the latter two tissues perhaps being more clearly compartmentalized, and may contain a predictable number or range of stem cells (Potten & Loeffler 1990). Such an epithelial patterning is not obvious in the lung, and the controls on stem cell frequency are unknown.

Tracheobronchial epithelium

Experimental access to the upper airway is easier due to its size and structure and so many reports relate to these tissues. Moreover, many pulmonary tumours are believed to arise here, particularly in the bronchus (Li *et al.* 1994). The mature epithelium consists of a pseudostratified layer of cells of four main types:

- Basal cells which lie on the basement membrane and which attach *via* hemidesmosomal junctions (Evans *et al.* 1993). The apex of these cells does not reach the airway lumen;
- Mucous secretory (or goblet) cells, which contain

vesicles of mucigen and possess an apical surface which contacts the lumen;
- Ciliated cells, comprising the majority of cells, which also have apical contact with the lumen, and are instrumental in the upward movement of mucus;
- Endocrine cells, having apical neuroendocrine granules which store such peptides as serotonin (5-hydroxy-tryptamine, 5-HT), calcitonin (CT), and calcitonin gene-related peptide (CGRP) (Sorokin & Hoyt 1989).

In addition, in the upper cartilaginous conducting airways of rabbits and hamsters are Clara-like cells, which are columnar and may have proliferative capacity (see below).

The production of ciliated and mucous cells from basal cell precursors via an intermediate cell type in the tracheobronchial epithelium (TBE) was first suggested in the 1880's by Drasch and Bockendahl (cited by Otani 1987). Reports from the past 30 years suggested that rodent lung growth during its lifetime was commensurate with the cell proliferative rate. The percentage of lung cells in the cell cycle (the growth fraction, GF) during intrauterine development is 100%, but soon into postnatal life this falls to 30%. At the same time, lung turnover time extends from 12 to 56 h. The cell cycle time rises from 12 to 15 h (Table 1). Foetal tracheal epithelial flash ^{3}H-TdR labelling index for basal cells is up to 70%. However, the label is not seen in ciliated or secretory cells for three days, although occasional earlier cells have been reported (Breuer *et al.* 1990). These data suggest that basal cells can produce the other two cell types (reviewed further by Wright & Alison 1984).

Proliferative data for human lung epithelia are severely limited. By contrast, human lung cellular ontogeny has been studied in greater detail (Cutz 1987). Here we see a paradox. The earliest cell to differentiate in lung epithelia is the neuroendocrine cell at 8 weeks of development, then preciliated and presecretory cells (10–11 weeks), and only by 14 weeks are prebasal cells recognizable. If the tracheobronchial basal cell is a stem cell, the *in utero* sequence may seem counter-intuitive. The developing tissue comprises many histologically undifferentiated cells (but maybe not at the genetic level) and their stem cell characteristics remain uncertain. However, any daughter cells could become recognizably differentiated. In tissue crossover experiments in early uterine lung morphogenesis, Hilfer (1996) reported a developmentally regulated committment to cell fate, so it is probable that a field of 'stemness' may operate. The above sequence of differentiation questions the origins of the neuroendocrine cells and neuroendocrine bodies. We need to understand how adult lung epithelial repair yields an almost reverse differentiation sequence and how both patterns relate to cell proliferation and differentiation in tumourigenesis.

Table 1. Some cell kinetic data relating to lung tissue in rodents

Epithelium	LI (%)	Cell cycle time (h)	DNA synthesis time (h)
Tracheobronchial	0.5–1	20.6	7.5
Wounded at 2d	28–30.5		
Bronchiolar	0.3–0.5	30	
Alveolar	0.2	22	7.7
Wounded at 2d	2–6		

Data taken from refs (Wright & Alison 1984; Plopper & Dungworth 1987; Adamson *et al.* 1990; McDowell *et al.* 1993).

Basal and secretory cells – can both act as stem cells?

Much evidence supports the basal cell as a stem cell. Breuer and colleagues (Breuer *et al.* 1990) recorded normal adult hamster ^{3}H-TdR flash labelling indices. In two types of basal cells, B1 and B2, distinguished by height of the nuclei above the basal lamina, the LI was 28% or 23%, respectively. This constituted a high percentage of labelled cells (50.6%) since basal cells only contributed 6.8% of all the epithelial cells present. Grain counts over nuclei only fell in B1 cells, and a cell cycle time of 20.6 *days* was estimated, with a DNA synthesis time $(T)_S$ of 7.5 h. Population B2 was suggested to contain other cell types, possibly secretory cells. There were also three categories of secretory cells identified, which were grouped by cytoplasmic mucigen granule content (S1, 0–4 granules; S2, 5 or more with visible cytoplasm; S3 with granules filling the cytoplasm). This probably reflects committment to differentiation into mucous cells. However, the LI of the secretory S1 cells was even greater than the basal B1, at 30.5%, while S2 and S3 indices were 6.4% and 0.0%, respectively, and did not alter over 14 d, yet that of the S2 group increased. This can be explained by a differentiation of basal cells into secretory cells. The initial LI of ciliated cells was 5.7%. Breuer and colleagues concluded that B1 basal cells can produce all cell types, while secretory cells, although still proliferative, do not show a stem cell nature. However, any flow of secretory cells into basal cells (should such mechanism for their being stem cells occur) may have been masked by the opposite flow from B1 cells into S1. It is probable that once a secretory cell commits to differentiation with a greater mucin content (i.e. S2, S3) that the likelihood of cell division greatly falls. This may be the pulmonary equivalent of the transit amplifying compartment suggested for the intestinal

crypt or skin (Hall & Watt 1989), with the clear difference that histological changes of some cells from basal to secretory can occur at the same time.

The above data retain the conundrum of the ciliated cell and its 5.7% LI. If these cells are not proliferative, as *in vitro* data from human (Wu *et al*. 1990) or hamster (Lee *et al*. 1984; Keenan 1987) tracheobronchial epithelial cells has suggested, then such a level of labelling infers that cells may progress from a very late-labelled basal or secretory type, through G2/M and into morphologically definite ciliated cells within an hour. This is improbable, and a small population of ciliated cells retaining proliferative capacity may exist in the hamster.

A flow cytometric approach to this problem has been reported (Johnson *et al*. 1990). Subpopulations of rat tracheobronchial cells were sorted, and more than 90% were accounted for. 10% were in G2/M, and of these 86% were mucous cells, with the rest basal cells. However, the overall proportions of the whole population differed from that found *in vivo*, since only 13% ciliated cells were sorted, whereas 40% exist in whole tissue. Clusters of ciliated cells were possibly removed during purification. Nevertheless, their colony forming efficiency (CFE) was estimated by plating them, serum-free, onto tissue-culture dishes. Unsorted cells had a CFE of 2.6%, and basal cells had a reduced CFE (0.6%) compared to secretory cells (3.4%). Reasons for these differences are not clear. Sorting may change the ability of cells to attach and spread, or may differentially damage cells. These data differ from those of Randell *et al*. (1991) who sorted the same cells by Griffonia (Bandeiraea) simplicifolia I (GS-I) lectin staining. Over 98% of lectin-positive cells were basal, and they stained with antibodies to cytokeratin 13, a basal marker. In addition, 98% of these cells did not stain with alcian blue-periodic acid schiff (AB-PAS), indicating a lack of mucin. The sorted basal cells displayed a CFE of 7.5%, over 10-fold greater than that seen above. On the other hand, the GS-I-negative cells gave an LI of 1.6%, but 30% of these cells could not be identified, and were probably a mix of secretory, ciliated with some basal cells (estimated at 2%). These results are hard to rationalize in terms of stem cell characteristics. The data may reflect the responsiveness of cells to their new environment, rather than something particular about their histogenetic potential. The answer may come from an experiment where categories of sorted cells are tested for their ability to repopulate a denuded trachea with a fully differentiated epithelium.

Stem cell lineage can be addressed by preparing subpopulations of tracheobronchial epithelial cells and seeding each in tracheas denuded of their own epithelium by freeze-thawing. This method ensures a closed system, which also retains a large amount of basement membrane material for good cell attachment. Tracheas grafted under the skin of nude mice are then studied for cell differentiation characteristics.

Several experiments of this type have appeared (Inayama *et al*. 1988, 1989; Johnson *et al*. 1990; Nettesheim *et al*. 1990; Randell *et al*. 1991). In a lectin sorting experiment (see above) (Randell *et al*. 1991) rat GS-1B4 positive basal cells produced a fully differentiated mucociliated epithelium by 20 d after inoculation into denuded tracheas. However, the GS-IB4 *negative* cells also produced this result. There may have been a basal cell residuum, or else mucous cells could have a stem cell potential. The resolution of these ambiguities awaits studies using purified secretory cells.

It should be noted that there are species differences in GS-1B4 expression: rats and mice have positive TBE basal cells, as noted above, whereas rabbits are negative. Alkaline phosphatase activity can be a marker for rabbit basal upper airway cells, although some activity is seen in Clara cells, type II pneumocytes and even endothelial cells, so care would be needed in its use. Ciliated cells appear negative (Inayama *et al*. 1995). Cytokeratin 14 is reported to be unique to the basal cells in human TBE (Broers *et al*. 1989) so this marker may have potential for determining the fate of similar experiments using human basal cell populations.

Nettesheim and colleagues have undertaken several experiments in a similar vein (Inayama *et al*. 1988; Inayama *et al*. 1989; Nettesheim *et al*. 1990). Rabbit tracheobronchial cells were obtained enzymatically with centrifugal elutriation to yield 92–94% basal cells. In this there were 2.4–2.9% Clara-like cells, 0.4–1.2% ciliated cells, and 3.4–4.1% undetermined cells. The basal cells produced a mucociliated epithelium over 4 weeks. They concluded that the basal cell is indeed a stem cell. These same cells in *in vitro* culture grew for up to 20 population doublings and displayed a CFE of 0.8–6%, which was similar to that of secretory cells. Basal cell clones were produced which also repopulated tracheas with a full range of cell types (Inayama *et al*. 1989; Nettesheim *et al*. 1990). This provides good evidence that basal cells possess the characteristics of stem cells. However, the ability of mucous cells to achieve this needs further experimentation.

Bronchiolar epithelium

Clara cells are cuboidal, projecting into the lumen more than the majority of the bronchiolar epithelium (Figure 1). They have much smooth endoplasmic reticulum, small

electron-dense nonmucin cytoplasmic granules and cytochrome P-450 activity (Nettesheim *et al.* 1990).

Early evidence from rats and mice (Wright & Alison 1984) suggested that Clara cells have a very low rate of proliferation. ^{3}H-TdR labelling index values were in the range 0.2–0.5%, with a cell cycle time of about 30 h. Adjacent ciliated cells did not label.

Sorting of rabbit Clara cells has been achieved by percoll density centrifugation and elutriation (Brody *et al.* 1987; Nettesheim *et al.* 1990). This yielded a population of 80–5% Clara cells, containing 4% type II pneumocytes and 0.6% basal cells. The cells expressed cytochrome P-450, were low cuboidal in shape, mucin-free and did not exhibit clonal growth. Inoculating denuded tracheas with only 20 000 Clara cells produced a low cuboidal simple epithelium of only Clara cells and ciliated cells. This contrasts experiments with whole tracheobronchial epithelial cells where a pseudostratified mucociliary epithelium was produced, which resembled that *in vivo*. The latter included the Clara-like (columnar) cell found in rabbit (and hamster) tracheas (Nettesheim *et al.* 1990). The histogenesis of this cell type is still uncertain, but probably derives from basal or secretory cells of the mixed mucociliary epithelium of the TBE, rather than from the true Clara cell, which is located more distally. Nevertheless, the conclusions about cell derivation from isolated Clara cells are that they can yield more Clara cells and ciliated cells but not basal or mucous cells. It could be argued that such sorting procedures could produce a change in differentiation potential, that a denuded trachea is not the normal location for a Clara cell, or that residual basal cells produced the epithelium. A fully mixed TBE inoculating population produced a mucoliliary epithelium, and this contained basal cells, as well as most other cell types (but not neuroendocrine cells – see below). The purified Clara cells had only 0.6% basal cells remaining. These 120 (0.6% of 20 000) basal cells would have had to both proliferate to confluence and change their differentiation programme to produce the observed result. This seems unlikely. A further 'intermediate' Clara cell type had much SER but no osmiophilic granules at the EM level (Hook *et al.* 1987). Whether these cells can repopulate a denuded trachea remains to be seen. They are probably intermediate in the differentiation of ciliated cells.

Further work needs to be done to investigate extracellular matrix effects on differentiation, since this may affect the outcome (see Alveolar epithelium, below). Subpopulations of TBE basal cells and true Clara cells should be compared when inoculated into a denuded *bronchiolar* tubule. Without this, it still seems probable that Clara cells are the stem cells for bronchiolar epithelium. The outcome of the above experiments suggested that differentiation can be independent of the subjacent mesenchyme, since the grafts were subcutaneous and so dermal cells would have invaded the stroma (Brody *et al.* 1987). More work is needed to define mesenchymal effects in the different lung areas.

Alveolar epithelium

The alveolar epithelium differentiates last *in utero*. Type I and II pneumocytes line the alveoli in a 1:2 ratio, yet the highly flattened squamous type I cells contribute 86% of the surface area for gas exchange (Weibel & Taylor 1996) (see Figure 1). Type I cell cytoplasm is only 0.15μ thick. Type II pneumocytes usually occupy alveolar corners, are cuboidal (Figure 1), and display abundant surface microvilli, mitochondria, rough ER, and a Golgi apparatus, as befits a secretory cell. They also express Mn-superoxide dismutase (Quinlan *et al.* 1995) and regulate surfactant production (Rannels 1996). Their hallmark is the presence of cytoplasmic lamellar bodies containing surfactant rich in disaturated phosphatidylcholine phospholipids (Plopper & Dungworth 1987; Rannels & Rannels 1989a), and they express tissue-specific genes such as surfactant proteins (SP) 1 and 2 (Hackett *et al.* 1996) and the EGF receptor (Minoo & King 1994). Surfactant production is exquisitely sensitive to glycogen mobilization (Rannels 1996), which is under the control of a testis-like isoform of phosphorylase kinase during development (Liu *et al.* 1996a). There is close association between cellular functions and the extracellular matrix, especially laminin and fibronectin (Rannels *et al.* 1987a, 1987b, 1989; Rannels & Rannels 1989a). In rodents the alveoli develop for several days after birth. During this period, the type II pneumocyte labelling index declines from 6% at 7 d to about 1% at 3 weeks, and in adults is 0.2% or less (Wright & Alison 1984).

Type I cells are highly sensitive to toxic gases (NO_2, O_3) and oxygen at high pO_2 (Rannels & Rannels 1989b). Flash labelling with ^{3}H-TdR after NO_2 treatment in rodents produces labelled type II cells – typically having a granular cytoplasm, and which stand proud of the adjacent squamous type I cells. After 2 days or so, the ^{3}H is seen in these latter cells. This is the founding evidence for type II cells to be progenitors of type I (Wright & Alison 1984). An early experiment (Gordon *et al.* 1992) had a curious result. Mice were labelled with ^{3}H-thymidine for 4 h and 1% of type I nuclei were labelled, compared to 10% of type II nuclei. No type I cells were ever seen in mitosis. With an S-phase about 7.7 h (Wright & Alison 1984; Uhal 1994) this suggests that

there is a population of type II cells blocked in late S-phase and which can respond quickly to local injury.

A similar observation was reported for the alveolar epithelium of rachitic rat neonates given vitamin D_3 (Edelson *et al*. 1994). Cultured alveolar type II cells from various developmental ages, or adult cells, responded to 1,25-dihydroxy vitamin D_3 by a rise in LI, and an increase S and G2/M cells, but without any change in cell number between 20 and 68 h after treatment. It is possibile that type II pneumocytes can enter S-phase and remain blocked in G2/M. However, there is a report (Clement *et al*. 1990) that type II cells can make unstable DNA *in vitro*, and may not divide. Such DNA synthesis confuses the use of ^{3}H-TdR as a proliferative index in these cells. How this relates to an *in vivo* situation is far from clear, but should caution against the overinterpretation of data using this label for type II cells.

Neuroendocrine cells

These are the first morphologically distinguishable cells seen in the developing foetal lung (8 weeks in humans, 12 days in hamsters), and have several neuropeptides including gastrin-releasing peptide (GRP – mammalian bombesin equivalent), MOC-1 antigen (N-CAM), 5-hydroxytryptamine (5HT, serotonin), protein gene product 9.5 (PGP-9.5), and calcitonin gene-related peptide (CGRP) among others, contained within electron-dense granules (dense-core vesicles) in the cytoplasm (See Table 2) (McDowell *et al*. 1993; Wang *et al*. 1996; Yeger *et al*. 1996). Neuroendocrine cells (NEC) develop in a centrifugal (proximal to distal) manner, the opposite to peptide YY (pYY) -positive NEC which begin development postnatally in the reverse direction, starting in the alveoli. pYY is absent from hamster pulmonary nerves (McDowell *et al*. 1994a). The developmental origin of the NEC is controversial. The Pearse hypothesis of a neuroectodermal origin, in particular gastrointestinal tract NEC (Pearse 1969), was subsequently refuted (Fontaine & LeDouarin 1977) by cell lineage tracing in chick-quail chimaeras, and allophenic male-female chimaeric mice (Thompson *et al*. 1990). Pulmonary origins are still unclear (Marchevsky 1990a; Marchevsky 1990b; McDowell *et al*. 1994a,b). There are both single neuroendocrine cells and clusters of morphologically similar cells in neuroendocrine bodies (NEB), only the latter being innervated (McDowell *et al*. 1993, 1994a, b). Both are epithelial, and may express many neuropeptides (Table 2) (Adriaensen & Scheuermann 1993; McDowell *et al*. 1993, 1994a, b; Polak *et al*. 1993; Wang *et al*. 1996; Yeger *et al*. 1996) depending on species and site, while some tumours may produce ectopic neuropeptides (Marchevsky 1990a, b). Human infant pulmonary neuroendocrine cells have been observed *in vitro* after enzymatic disruption of tracheal and bronchial tissue. NEC stained positively for GRP, 5HT and MOC-1. There were single cells as well as clusters, reminiscent of NEB, which overlaid the other epithelial cells (Yeger *et al*. 1996). Foetal rabbit and human foetal and neonatal pulmonary epithelium were found to express GRP receptor (GRP-R) mRNA at a maximum during the canalicular branching phase of development, when the epithelium was undifferentiated and tubular. Some GRP-R was seen in the stroma, but at lower levels. By contrast the NEC and NEB showed expression of GRP and not the receptor. Foetal rabbit NEC were seen to accumulate in culture after exogenous GRP stimulus (Spiers *et al*. 1993).

Normally there is no proliferative activity in NEC (Hoyt *et al*. 1990; Montuenga *et al*. 1992), although this may not be absolute (Stahlman & Gray 1984). BUdR exposure on embryonic day 12 in hamsters revealed no labelled neuroendocrine cells the following day. Instead, the adjacent epithelium had labelled nuclei (McDowell *et al*. 1993, 1994a, b). In similar studies of rabbit foetal lungs, NEC and NEB did not show MIB-1 (Ki67) staining, an indication that they were not in the cell cycle. Adjacent epithelial cells were usually MIB-1 positive (Spiers *et al*. 1993). Taken together, these data strongly suggest a

Table 2. Neuroendocrine cell peptides

5-HT (serotonin)*	MOC-1 (N-CAM)
Acetylcholinesterase	Neurone-specific enolase
Bombesin/Gastrin Releasing Peptide	Pancreatic secretory trypsin inhibitor
Calcitonin	PGP 9.5
Calcitonin gene related peptide	Pituitary adenyl cyclase activating peptide
Cholecystokinin	pYY†
Chromogranins A-C	Somatostatin
Enkephalin	Substance P
Leu-7 (HNK)	Synaptophysin
Ectopic neuropeptides in tumours	
Adrenocorticotrophic hormone	Motilin
Corticotrophin releasing peptide	Neurotensin
Glicentin	Vasoactive intestinal polypeptide
Growth hormone releasing peptide	

*Adult rats and mice are 5-HT negative; other foetal and neonatal mammals have raised levels

†pYY$^+$ neuroendocrine cells are always negative for 5-HT, CGRP and calcitonin.

Neuroendocrine cells may not simultaneously express all neuropeptides.

From references: (Adriaensen & Scheuermann 1993; McDowell *et al*. 1993, 1994a, 1994b; Polak *et al*. 1993; Wang *et al*. 1996; Yeger *et al*. 1996).

paracrine effect for GRP, and the significance may extend to a role in tumour development where ligand and receptor are often prominent in small cell lung cancer.

However, the nature of NEC precursor cell(s) remains obscure. Hoyt and colleagues (Hoyt *et al*. 1993) reported 43% of either ^{3}H-TdR or BUdR localization in the proximal 2/3 of developing hamster lungs was within 20μ of NEB. In addition, the spread of autoradiographic nuclear silver grains was broad, and they inferred that the NEC precursor (stem?) cell was probably next to the NEB. NEB cells may actively deter the surrounding epithelium from differentiating, a feedback-inhibition mechanism. A similar conclusion about stem cell positions has been expounded (Montuenga *et al*. 1992). It is unclear whether these juxta-NEB cells are stem cells for all lineages in the whole lung, or merely facilitate local cell differentiation (i.e. tracheobronchial, bronchiolar, or alveolar). If cells adjacent to NEC or NEB can be purified, then the reinoculation of tracheas as described above should answer the question. The importance of a 'stem cell niche' is discussed below.

In the experiments seeding different cell types into tracheas, and in reports on pulmonary responses to injury, there was no evidence presented on neuroendocrine cells. Their differentiation may be rare, or there were inappropriate signals. The search for these cells is important when considering the derivation of lung tumours (Sidhu 1979; Wright & Alison 1984; Gatter *et al*. 1985; Greenberg 1987; McDowell *et al*. 1987, 1994a, b; Marchevsky 1990a; Wright 1990; Gatter & Dunhill 1992). Of possible significance here is the role of GRP in pulmonary cultures in raising the numbers of NEC (Spiers *et al*. 1993).

Pulmonary NEC 'hyperplasia' may result after carcinogen treatment in hamsters given supranormal oxygen (Sunday *et al*. 1994). The tobacco smoke constituents 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK) or diethylnitrosourea (DEN) induced increases in NEC numbers. Those occurring with NNK persisted for 20 weeks, while the DEN-stimulated proliferation regressed spontaneously. C-myc mRNA increased in the non-NEC epithelium of both the oxygen plus chemical-treated groups, without changes in c-raf, k-ras or p53 expression. Proliferating cell nuclear antigen (PCNA) was restricted to a mostly non-NEC subset of epithelial cells. It was inferred that an increased neuroendocrine differentiation caused the rise in NEC, rather than proliferation, and that paracrine events dependent on the local peptide profile would determine the phenotypic outcome.

The above discussions are based on experiments where the pulmonary tissue was unperturbed by toxic gases, chemicals or fibres, any of which may provoke a wound healing response. The isolation of cell subpopulations by enzymes and differential centrifugation could result in injury. Notwithstanding this objection, the preceeding reports have been discussed separately from those about the *sequelae* of several damaging procedures on the lineage potential of survivors.

Lung stem cells in injury

Tracheobronchial epithelium

There are many papers which describe the effects of several toxic insults to the lung. Injury from mechanical (McDowell *et al*. 1990; Kuboi *et al*. 1992; Horiba & Fukuda 1994; Kanno & Fukuda 1994; Shimizu *et al*. 1994), gaseous (Adamson *et al*. 1990; Tanswell *et al*. 1990; Roemer *et al*. 1993; Absher *et al*. 1994; Bowden *et al*. 1994; Bui *et al*. 1995; Lee *et al*. 1995; Rajini & Witschi 1995), fibre or particulate (Woodworth *et al*. 1983; Marsh & Mossman 1991; Gibbs & Wagner 1992; Lesur *et al*. 1992a, b; Janssen *et al*. 1994, 1995a, b; Marsh *et al*. 1994; Begin *et al*. 1995; Driscoll *et al*. 1995; Mossman *et al*. 1995; Quinlan *et al*. 1995; Timblin *et al*. 1995; BeruBe *et al*. 1996a, b; Dufresne *et al*. 1996; Melloni *et al*. 1996; Treadwell *et al*. 1996), and chemical insults (Fukuda *et al*. 1985; Adamson *et al*. 1990; Radosevich *et al*. 1990; Simons *et al*. 1991; Barrow *et al*. 1993; Roemer *et al*. 1993; Madtes *et al*. 1994; Ooi *et al*. 1994; Roggeband *et al*. 1994; Van Benthem *et al*. 1994; Shorten *et al*. 1995; Spencer *et al*. 1995a, b, 1996; O'Hare *et al*. 1996) or vitamin A deficiency (Chopra *et al*. 1990; Niles *et al*. 1990; Zhang & McDowell 1992; Inayama *et al*. 1996; Miller *et al*. 1996) have been reported recently. Fibre and chemical injury were reviewed by Mossman *et al*. (1996).

Tracheal responses to mechanical injury

Shimizu *et al*. (1994) injured adult male rat tracheas and investigated adjacent cells. Within 12 h, wound edge cells flattened and migrated over the denuded area and became poorly differentiated. 23–24% of epithelial cells were in mitosis at 24 and 48 h, and this fell to 1.8% at 72 h, when control rats had a mitotic index (MI) of 0.38%. A multilayered epithelium was restored at 48 h, and by 72 h this had become pseudostratified with a majority being secretory cells, but these retained some basal cell characteristics (cytokeratin 14^{+}, Griffonia simplicifolia isolectin B4^{+}), which then declined as differentiation proceeded. Poorly differentiated cells also existed in control epithelia (8.3%). Secretory mucous cells

disappeared 12 h after injury. By 24 h poorly differentiated cells rose to 88% of the cells present, with 10.3% secretory cells. By 48 h the total cell number was restored, but ciliated cells were not seen until 72 h. The new mucous cells also retained some basal markers, which reduced with time. The complete mucociliary restoration took 14 d. The poorly differentiated cells still had some mucous cell characteristics. It was suggested that some basal and secretory cells 'dedifferentiated' into poorly differentiated cells, which proliferated and developed into new basal and mucous cells. The origin of the ciliated cells was unclear, since no cells were seen which coexpressed ciliated with either basal or secretory markers.

Further experiments described mechanically injured rat tracheas (Horiba & Fukuda 1994). Over a 3-week period the expression of the fibronectin receptor (integrin $\alpha 5\beta 1$), vinculin and actin were followed. Their appearance correlated with BUdR incorporation, which peaked at 18 h in 40% of epithelial cells (fibroblasts peaked at 15% LI, but not until 2 d). LIs became normal between 10 and 14 days. It was concluded that plasma, not cellular, fibronectin was important in stimulating cell division and migration.

The role of tenascin (TN), an extracellular matrix protein, in tracheal wound healing was reported by Kanno and colleagues (Kanno & Fukuda 1994). Normally absent, TN subjacent to the epithelium rose to a maximum 18 h after wounding, which coincided with maximum BuDR labelling. Thereafter TN deposition declined except in the granulating stromal tissue. Almost no epithelial cells incorporated BuDR if they adhered to fibrin, but those over the TN^+ stroma were labelled. It was concluded that fibroblasts produced TN and this altered epithelial behaviour during migration and proliferation over a fibronectin matrix.

Tenascin may play a role in the positioning of stem cells. Normal brain tissue does not divide, but a small area in the subventricular zone of the lateral ventricle in the forebrains of adult mammals contain dividing cells. These take up BUdR, express nestin and are underlain by tenascin-C and chondroitin sulphate proteoglycan. Tenascin-C levels rise around injury in brain, and may play role in facilitating cell movement after such events (Gates *et al*. 1995).

The role of collagen type I gels on the ability of rat tracheal epithelial cells to adhere and form differentiated cultures was investigated by Davenport & Nettesheim (1996b). Cell proliferation was not affected by the substratum, but colonies were larger, and had more mucous cells on collagen. Ciliated cells were even more sensitive to the lack of a substratum, and were sparse unless the precursor population was seeded onto collagen. Refinements to such culture systems have established that cilia production is increased in the absence of growth factors (Ostrowski *et al*. 1995) or liquid submersion (Ostrowski & Nettesheim 1995). Both these results point to the necessity of prior steps (growth stimuli, matrix deposition) in the determination of ciliated cells to be fulfilled, and this emphasizes the lineage dependence of the process. The culture of rat tracheobronchial cells is accelerated in the presence of EGF, but ciliated cell production is lower. EGF removal decreases mucous production and MUC-5 expression, but has little effect on mucous cell number once they form (Guzman *et al*. 1995). Secretion of ECM proteins by RTE cells was found to be lower on collagen gels than on plastic (Davenport & Nettesheim 1996a, b). Thus expression of collagen IV $\alpha 1$ chain, fibronectin, thrombospondin-1 mRNA were all lower in cultures on collagen I gels than on plastic. Laminin C1 and B1 chains were not affected. An important finding was that the appearance of ciliated cells was greatly increased on gels, which was explained by differentiation of precursor cells, rather than attachment events of preciliated or ciliated cells onto the gel. The authors concluded that the ciliated cell is more dependent on ECM interactions for function than the mucous secreting cell, and that time spent on the ECM is important.

Foetal (E15) rat organ cultures were studied for the effects of proteoglycan and collagen synthesis on branching morphogenesis (Matsui *et al*. 1996). Inhibitors of proteoglycan synthesis resulted in a static lung size, with larger air spaces and less mesenchymal tissue and fewer proximal branching events. In contrast, collagen synthesis inhibitors prevented distal branching.

Tracheal responses to gaseous injury

Adamson & Bowden (1974) and Adamson *et al*. (1990) described the likely lineage of alveolar cells. Supranormal oxygen, 90% for 6 days, destroyed most murine type I pneumocytes. The epithelium recovered after return to normoxia, during which the LI of type II cells rose from 0.03% to 2% at 24 h (Adamson *et al*. 1990), but there was no simultaneous rise in the LI of underlying fibroblasts. There was a similar delay to that noted above (Horiba & Fukuda 1994). Labelled nuclei began to appear in type I pneumocytes over the next few days (Adamson & Bowden 1974). It was observed that direct type II cell-fibroblast contact was made through a discontinuous basal lamina, but no contact was made by type I cells (Adamson *et al*. 1990; see also Fukuda *et al*. 1985). Contacts rose from 0.48 contacts per type II cell to 0.65 after 3 d of hyperoxia, while numbers of type II cells

displaying a continuous basement membrane declined from 40% to 29%. It was concluded that direct contact with fibroblasts helped to restrain type II cell division, and those cells inhibited underlying fibroblasts from proliferating by secreting PGE_2. Type I collagen fibrils underlying type II cells somehow helps maintain their cuboidal shape. In parallel studies, butylated hydroxytoluene (BHT) and bleomycin-induced type II cell damage persisted, and there were reduced epithelial-fibroblast contacts and increased labelling indices. The mRNA levels in epithelia for TGF$-\alpha$ and the EGF receptor peaked at 4 and 7 days, respectively, after bleomycin damage to type I cells (Madtes *et al*. 1994).

The cross-talk between mouse lung epithelium and fibroblasts in the control of repair after a 6 day exposure to 90% oxygen was also reported (Bowden *et al*. 1994). Lung explants showed toxic effects on type I pneumocytes, with a drop in 'cuboidal' (possibly type II pneumocytes) cell multiplication compared to controls. At the same time the level of hydroxyproline increased, consistent with new collagen production, while fibroblast proliferation also increased. Treatment with L-azetidine carboxylic acid (LACA), a proline metabolism inhibitor, prevented the enhanced fibroblast proliferation, but cultures still fibrosed without epithelial repair. LACA inhibited the proliferation of pure lung fibroblasts cultures, but not epithelial cells. It also prevented a growth stimulus seen when epithelial cells were exposed to fibroblast-conditioned medium. It was concluded that there was close dependence between these cell types during repair, and simply preventing fibrosis may not promote lung recovery.

Rajini & Witschi (1995) gave Sprague-Dawley or F344 rats several exposures to ozone and measured the LI after a BuDR minipump infusion. Epithelial LI did not alter in the nasal septum or trachea. Terminal bronchioles were the most sensitive cells, for which the LI was proportional to the product of ozone dose and time. Unfortunately no cell types were identified in this study. It was probable that those bronchiolar cells proliferating were Clara cells, but this needs confirmation. Strain differences were few, and it was concluded that intermittent high doses of ozone result in greater damage than lower continuous ones, and that tissue adaptation occurs. Any future study should analyse pneumocyte responses and identify the reactive cell types in the tissue.

Roemer *et al*. (1993) reported on the damage produced by inhaling formaldehyde (up to 20 p.p.m.) and acrolein (up to 0.6 p.p.m.) in young adult male Sprague Dawley rats. LI values rose in nasal and tracheal epithelia after 2 p.p.m. formaldehyde, while 0.2 p.p.m. acrolein produced additional LI rises in the peripheral lung. Unfortunately this report did not identify the cells responsible for the changs in labelling, but it did show that there are mechanisms for the airway compartments to respond to damage by cell production.

There have been reports on type II pneumocytes exposed *in vitro* to ozone (Tanswell *et al*. 1990) or hyperoxia (Cazals *et al*. 1994). In the first, plasma or lung lavage fluid from ozone-treated rats was used to stimulate 20 d foetal rat type II cells. Both sources of fluid produced a rise in labelling index, and a protein of 38 kD/pI 6.45–6.75 was isolated, which was heat-stable, and which affected epithelial cells but not fibroblasts. The source of the protein was not found, but the results suggest that paracrine effect(s) may contribute to stimulating type II cell responses to injury, in addition to the effects of direct cell-cell contact noted above.

Rat type II cells immortalized with the SV40 large T antigen were exposed to hyperoxia *in vitro* (Cazals *et al*. 1994). The hyperoxia stopped cell proliferation, while the mRNAs for insulin-like growth factor (IGF), its binding protein IGF-BP-2 and its type 2 receptor (IGFR-2) all rose, as did TGF-β expression. Normoxia reversed these changes. The observations increase the list of factors upregulated after cell injury, and suggest a complex network of effects that control wound healing.

Adult type II pneumocytes from rats were studied by FACS analysis after 48 h 100% oxygen exposure followed by recovery in air (Bui *et al*. 1995). There was a 3–4-fold rise to a peak of 13.6% G2/M cells at 2 days of recovery. The S phase fraction rose earlier by three-fold to 4.3% of cells at one day recovery. These alterations had parallel increases in BUdR incorporation (S-phase), PCNA expression and in the induction of cyclins A and D1 in the lungs. In addition $p33^{cdk2}$, $p34^{cdc2}$, cdc2-associated histone H1 kinase, and to a lesser degree $p34^{cdk4}$ proteins were induced in recovering lungs. It is clear that type II cells follow the usual cell cycle dependent steps familiar for other cell types. Further cyclin details are to be found in recent reviews (Reed 1992; Sherr 1993).

In a more kinetic experiment (Lee *et al*. 1995) rats were exposed to O_3 (0.55 p.p.m.) or NO_2 (10.8 p.p.m.) for 7 daily 8-hour periods. These concentrations approximate to those found in urban settings. Cumulative LI values were estimated after BUdR infusion by minipump. There was a linear relationship between centriacinar and terminal bronchiolar LI for the collected data from all the experimental groups (Controls, O_3, NO_2, both gases). The absolute values for labelling were greater in the terminal bronchioles than the centriacinar region when exposed to NO_2 or both O_3 and NO_2. This was despite the counting of only epithelial cells in the former and all

cell present in the latter areas (including endothelial and stromal cells). The sensitivity of the centriacinar region to the toxic effects of ozone was clearly revealed, but the whole distal lung seems to react. Although not specified in this report, the cells responding were almost certainly the type II and Clara cells.

Mineral fibre damage to the lung

Inhaled particles reach different locations in the lung depending on their size. Above 5μ, the conducting airways filter dusts, where ciliated and mucous cells cooperate and waft the particles upwards. Particles below 5μ can reach the alveoli, and if trapped there may have long-term damage potential (Gibbs & Wagner 1992). Phagocytic macrophages or polymorphonuclear leukocytes (PMN) are recruited but most particles are indigestible and result in the cell death and a repeated stimulus, from which fibrosis or other pathology may ensue. The finest dusts reach the alveoli where type II pneumocytes release excessive levels of surfactant which can fill the lumen (Gibbs & Wagner 1992). Toxicity of asbestos fibres is broadly proportional to their physical size (length to diameter, or aspect, ratio), with a tumourigenic gradient decreasing from crocidolite (blue) asbestos to amosite (brown) to chrysotile (white). Particles with aspect ratios below 3:1, such as riebeckite (same chemical formula as crocidolite) or polystyrene beads, are not strictly fibres, and are less toxic (Janssen *et al*. 1994).

A recent rat study found that over half of inhaled christobalite (reactive silicon dioxide, particle size 1μ) was retained by macrophages in the lungs for over a year after two 4-day exposures. Macrophage numbers peaked after 8 weeks. PMN and lymphocyte numbers also remained high for several weeks. TGF-α production by the macrophages increased (other cells not tested), and this was estimated at 180 nM in lung epithelial lining fluid. Two minimally reactive crystalline dusts had no effect on TGF-α secretion (Absher *et al*. 1993). TGF-α is a known mitogen for type II pneumocytes (Ferriola *et al*. 1991; Chess *et al*. 1994; Korfhagen *et al*. 1994; Ryan *et al*. 1994). It was concluded that the persistent stimuli from the dust and phagocytes led to progressive alveolar remodelling resulting in silicotic nodules and a loss of functional tissue. This mechanism may augment carcinogenic effects of other long-lived toxic agents, and suggests an interaction between the macrophage and type II cell in these processes.

Bowden & Adamson (1984) instilled the tracheas of mice with a single exposure to smaller (0.3μ) silica particles. Damage and inflammatory cell infiltrate was studied. By 24 h, acute focal necrosis in type I pneumocytes was evident, with silica particles contained within surviving type I cells. Basement membrane was greatly denuded, with abundant fibrin deposits. However, epithelial restoration was quick. Type II cells had a maximal LI after 2 days, but the inflammatory reponse continued for 20 weeks, when predominant macrophages were accompanied by high levels of PMN. Silicotic granulomas that had appeared within a week also persisted, and even though hydroxyproline deposits (neo-collagen) rose two-fold, the amount of scarring at the end was minimal. This contrasts with the above data for rats receiving the larger reactive silica christobalite, where long-term nodules persisted, with loss of lung function (Absher *et al*. 1993). There may be an important relationship between particle size and the tissue response, especially the role of macrophage-derived TGF-α on tissue repair. Whatever the mechanism, it seems clear that repopulation of damaged alveoli is the main remit of surviving type II pneumocytes.

Human and sheep lungs exposed by inhalation to occupational or experimental silica produced inflammatory cell infiltrates very similar to those in rodents (Lesur *et al*. 1992b). Broncho-alveolar lining fluid from silicon dioxide-exposed sheep stimulated the growth of type II cells *in vitro* in a ^{3}H-TdR assay, even 2 years later. Human lung fluid was also stimulatory. Two active peaks were obtained from the sheep lung fluid by sephadex G-50-heparin column chromatography, which were consistent with platelet-derived growth factor (PDGF) and acidic fibroblast growth factor (aFGF). Several other growth factors were found to stimulate rat type II cells *in vitro*: endothelial cell-derived growth factor (ECGF), IGF-1, TGF-α, and EGF. A synergy was noted between PDGF and ECGF, which could be amplified by IFG-1, TGF-α or EGF. Thus the control of type II repair processes depends on the important interplay between growth factors and cytokines (Table 3).

Evidence on the role of fibres in the stimulation of lung epithelial repair, and possibly carcinogenesis, was reported by Janssen *et al*. (1994). Hamster tracheal

Table 3. Growth factors active on lung epithelial cells

Mitogens	bFGF	GRP/Bombesin	PDGF-BB
	Collagen I	IGF-1	TGFα
	EGF	KGF	Vitamin D
	Fibrinogen	Lipocortin	
Differentiation factors	ECM	Steroids	
	Glucocorticoids	TGF-β	
	Retinoids		

From references: (Jetten 1991; Nettesheim *et al*. 1992; Absher *et al*. 1993; Barrow *et al*. 1993; Gray *et al*. 1993; Joiakim & Chopra 1993; Edelson *et al*. 1994; Mouhieddine *et al*. 1994, 1996; Ulich *et al*. 1994).

epithelial (HTE) cells and rat pleural mesothelial cells were exposed to fibrous particles *in vitro* – crocidolite, man-made vitreous fibre-10 (MMVF-10), refractory ceramic fibre-1 (RCF-1), erionite, riebeckite and polystyrene beads. In HTE cells all particles with an aspect ratio greater than 3 (i.e. true fibres: crocidolite, MMVF-10, RCF-1, erionite) induced the expression of c-jun mRNA, while only the first failed to induce c-fos mRNA. However, when hydrogen peroxide was present, this too was induced. Unfortunately, which cell types were ingesting the particles was not reported. Erionite, a carcinogen producing mesotheliomas in rats and humans, caused a significant induction of c-jun and c-fos expression in rat mesothelial cells. Non-fibrous particles had no effects, indicating the importance of geometry on this process.

Several recent authors have reported on the effects of mineral fibres (Janssen *et al.* 1995a, b; Mossman *et al.* 1995; Quinlan *et al.* 1995; Timblin *et al.* 1995; BeruBe *et al.* 1996b) and dusts (Begin *et al.* 1995; Driscoll *et al.* 1995; Melloni *et al.* 1996) on lung epithelia and mesothelia (BeruBe *et al.* 1996a). A review of the interrelationships between smoke, asbestos and cancer has also appeared (Mossman *et al.* 1996). Aspects of these pertinent to the stem cell debate are briefly considered.

The asbestos form crocidolite is capable of inducing active oxygen species (AOS: $O_2^{-\cdot}$, $OH^{\cdot}$) in several cells, including human type II pneumocytes and hamster tracheobronchial epithelial cells. Increases in mRNA for ornithine decarboxylase, c-fos and c-jun as well as superoxide dismutase (SOD) were seen soon after AOS exposure, and these rises were prevented by antioxidants (Mossman *et al.* 1995; Timblin *et al.* 1995). Similar inductions of c-fos and c-jun were reported for rat mesothelial cells, where apoptosis was also induced by crocidolite, and in type II cells and fibroblasts (BeruBe *et al.* 1996a).

The interplay of macrophages with type II cells was explored by Melloni *et al.* (1996). Human alveolar macrophages were exposed to silicon dioxide *in vitro*, and the conditioned medium applied to rat foetal (day E19) type II cells in culture. Fractionation of the CM revealed four activities, which were similar to PDGF, IGF-1 and FGF, as defined by cross-reacting antibodies to these factors. The type II cells reacted mitogenically to each fraction. Aluminium-treated silica had no effect in this system, indicating its possible protective effect. In contrast, sheep exposed *in vivo* to silica by inhalation showed no alteration in their fibrosis when later treated with aluminium lactate aerosol (Begin *et al.* 1995). This suggests there is little scope for amelioration of silicosis after the event.

Lung damage caused by chemicals

Several reports indicate the effects of different types of compounds on lung tissue. Continuous iv infusion (Simons *et al.* 1991) or bolus tracheal instillation (Ooi *et al.* 1994) of bacterial (Escherishia coli) lipopolysaccharide (LPS) endotoxin into rats was performed. The inflammatory cell infiltrates differed in their time-courses, with a continuous infusion resulting in a peak PMN population of 59% at 60–72 h, while a bolus dose brought PMN to a tracheal maximum at 8 h, when the greatest oedema occurred. By contrast, the alveolar PMN peaked at 4 h, and had disappeared by 12 h. Histologically, trachea and alveoli were little changed by an LPS bolus, whereas bronchioles showed a doubling in ciliated cell height, and a stimulation of undefined 'progenitor' cell proliferation at 24 h, and finally becoming pseudostratified with more Clara and ciliated cells. By contrast, a continuous LPS infusion caused alveoli type I cell damage leading to type II cell proliferation, which peaked at 48–72 h. Thus there is a clear, but unexplained, difference between the effects of LPS which depends on the route of administration: exogenous LPS affects bronchiolar cell production, iv LPS affects type II cells.

The toxic effects of smoke and its constituents are well documented (Mossman & Eastman 1987; Trevisani *et al.* 1992; Cook *et al.* 1993; Mulshine *et al.* 1993). A few reports are summarized below to illustrate the effects on pulmonary epithelial repair.

Barrow and coworkers (Barrow *et al.* 1993) compared the tracheal responses of fasted 3–4-year-old sheep to cotton smoke in animals which received an aerosol containing EGF with PDGF 72 h after injury. One day later, both groups showed a reduction in basal cell numbers. However, control tracheas were still denuded of epithelial cells at 10 days, whereas treatment with EGF/PDGF accelerated their recovery both in terms of cell number and differentiation. Tracheal mucous glands showed some cell proliferation, which may have contributed cells to the luminal surface.

In a different approach, Van Benthem and colleagues (Van Benthem *et al.* 1994) gave rats, mice and hamsters intraperitoneal 4 (methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco smoke constituent. The DNA adducts O^6-methylguanine and 7-methylguanine were detected by immunocytochemistry. By 6 h the upper airways were positive, in particular the nasal sustentacular and Bownman's gland cells. Serous glands, tracheal basal cells, bronchiolar Clara cells, alveolar type I and II cells all showed positivity. The staining was broadly proportional to cytochrome P-450 expression, although exceptions were rat Clara cells and

mouse type II cells, which had no detectable adducts. This may be due to technical or species differences and requires clarification. Nevertheless, several progenitor cells of the different airway compartments were direct targets for this carcinogenic chemical. It was also evident that the adduct pattern went beyond the 'target' tissue (i.e. included the pancreas), but that other tissues could be spared. Thus no staining was found in the oesophagus, small intestine, colon, kidney or prostate. This implied that other factors than DNA methylation must be considered when predicting oncogenic effects.

Klein-Szanto and colleagues (Klein-Szanto *et al.* 1992) described the effects of formaldehyde and the smoke carcinogen benzo-(a)-pyrene diol-epoxide (BPDE) exposure to human full-term foetal or infant lung epithelial cells seeded in denuded rat tracheas and xenotransplanted in athymic mice. Such tracheas reform a mucociliary epithelium by 4 weeks (see above). ^{3}H-thymidine LIs increased with formaldehyde dose, and a focal dysplasia or metaplasia was seen in the epithelium, in which even higher LIs were observed. Erosion and inflammation were also seen. When tracheas were treated with BPDE and formaldehyde together, or sequentially, similar results were found, which suggests that no additive or synergistic damaging events occurred.

Organ cultures of tracheal tissue from hamsters and rats, both sensitive to tumour induction by BP, have been studied (Roggeband *et al.* 1994). Two days exposure (5 μg/mL) resulted in diolepoxide (BPDE, carcinogen) formation on deoxyguanosine (BPDE-N^{2}dG) in both sources of tissue, but hamsters were 10 times as sensitive as rats. Only 60% of rat adducts were BPDE-N^{2}dG, but these persisted after BP removal, whereas the hamster adducts declined with time. Rats also had BPDE-deoxyadenosine adducts. Hamsters, but not rats underwent unscheduled DNA synthesis during BP treatment, and this too declined on BP removal. Labelling indices rose in both epithelia after BP treatment, but on different time scales: in hamsters, LI rose to 15–20% after 4–6 days BP exposure, while in rat tissues it peaked at 6–12% during BP treatment, and fell to below 1% at 4–6 days. These data show the potential significance of cell proliferation in 'fixing' carcinogens in the nucleus, but also suggest that other factors are important in carcinogenesis than the occurrence of DNA adducts.

The insecticide paraquat is highly toxic to lung tissue, and a recent study in cynomolgous monkeys described the damage which it produced. Paraquat was administered by subcutaneous injection (10 mg/kg). Two days later, type I cells had disappeared from alveoli, and there was much fibronectin deposited in the alveolar lumens. With other noxious agents type II cells migrate over the discontinuous basement membrane (Adamson *et al.* 1990). In this case fibroblasts moved through the gaps and a fibrotic reaction followed, which was well established at 3–4 weeks. The epithelium survived in foci, but it became metaplastic and squamous, similar to other repair situations (Klein-Szanto 1987; Nasiell *et al.* 1987). The presence of 'bronchiolar' cells was recorded, possibly Clara cells migrating into the denuded alveolar spaces. Although type II pneumocytes were seen next to the intra-alveolar fibrosis, they did not succeed in repopulating the area, and there was a loss of functional tissue. These results again indicate the role of type II cells, and also possibly Clara cells, in alveolar repair after paraquat. Whether different chemicals can stimulate Clara cell migration into alveoli is unclear, but the possibility needs to be considered when tumour 'stem' cells are discussed (see below). In this context, a report on the effects of pneumonia in rats concluded that both Clara and type II pneumocytes shared a common precursor cell (Rhodes *et al.* 1990).

DNA strand-breaking agents such as H_2O_2 and smoke have been studied in human TBE cells in culture (Begin *et al.* 1995; Spencer *et al.* 1995a, b, 1996). It was found that most damage was attributable to OH$^{\bullet}$ radical attack. H_2O_2 affected adenine residues, while the damage was to xanthine and hypoxanthine after smoke. These experiments indicate the critical and direct effects on DNA in the cells lining the upper airway.

Mammalian vitamin A deficiency (VAD) is characterized by tracheobrochial epithelial hyperplasia, stratification and squamous metaplasia (Chopra *et al.* 1990; Lancillotti *et al.* 1992). It was recently reported (Zhang & McDowell 1992) that an important feature in the hyperplasia was the presence or absence of inflammation. Hamster tracheal tissue was essentially normal (pseudostratified) if inflammation was absent, but where inflammation existed cells were hyperplastic, dividing at all strata except the most superficial. Mitotic (MI) and BUdR labelling (LI) indices of vitamin A deficient mucous cells in these two areas were very different: without inflammation, MI was low (0.03% of all cells), but when inflammation was present, the MI was maximal at 1.59%. It was then hard to distinguish basal from secretory cells, and these were grouped as 'nonciliated'. There was no effect of VAD on basal cell proliferation, leading to the conclusion that a basal cell 'hyperplasia' is really the reduction in other cell types. This study highlights the importance of detailed cell identification and taking as many factors into account as possible in reaching conclusions. Unfortunately not reported in this study, it will be interesting to follow neuroendocrine cell differentiation in this system. Whatever their role, it may be

inferred that vitamin A deficient smokers have a higher risk of tumourigenic 'hits' than a vitamin-sufficient one (Cook *et al*. 1993). In support of this was the report that vitamin A or retinol inhibit the airway expression of one potent mitogen for lung epithelial cells, TGFα (Miller *et al*. 1996). Removal of this paracrine inhibition in vitamin A deficiency allows excess TGFα production which may assist in squamous metaplasia and may allow proliferation in damaged cells to be 'fixed'. The degree of inflammation could be significant in promoting such events.

Further evidence in support of this came from Inayama and colleagues (Inayama *et al*. 1996), who seeded rabbit TBE or purified basal cells into denuded tracheas grafted into nude mice to investigate the role of vitamin A deficiency on squamous metaplasia. VAD mice showed atrophic epithelial grafts, with focal metaplasia, which was reversed in VA treated mice. Ciliated cell numbers altered dramatically under VA influence: 1–3% in VAD, but over 18% in VA replete mice. No changes were noted in basal cell numbers in either epithelium. The authors concluded the basal cell has stem cell characteristics, and is capable of undergoing squamous differentiation in the absence of vitamin A.

Growth regulation in lung epithelia

The last decade has seen much data appear on the roles of growth factors, cytokines and extracellular matrix components on growth and differentiation of many lung epithelial cells, with much information coming from *in vitro* studies. Many results describe the responses of several cell types, and it is not surprising to see the following as important players: tracheal basal and mucous cells, Clara cells from bronchioles and type II alveolar cells. Neuroendocrine cells are almost unrecorded in these studies, probably due to their scarcity and the difficulty of isolation. The NEC may well control their locality using paracrine factors, and there are reports of epithelial cells treated with neuropeptides which could model these effects (Sethi *et al*. 1992; White *et al*. 1993). The number of factors relevant to lung functions is increasing, and a summary is given in Tables 2 and 3.

The ability to respond to many of these factors is due *a priori* to the expression of ligand receptors and their downstream second messenger pathways, and how these are coordinated (or not) in repair and carcinogenic processes is not always known. The restraining influences of other factors, such as TGF-β, should be borne in mind (Pelton & Moses 1990).

Embryonic rat lung epithelial cells have been reported to secrete a factor which regulates the amount of subjacent stroma. Thus the conditioned medium (CM) from cultures of cells from the canalicular stage of lung development (E19–20) could inhibit pulmonary (but not dermal or intestinal) fibroblast growth. CM from the pseudoglandular (E18) or saccular (E21) stages had no such effects. The factor was labile in the presence of serum (Caniggia *et al*. 1995).

Some growth control is afforded by the simple movement of the lung tissue. Thus reports on the effects of mechanical strain on foetal (E19) rat lung epithelial cells grown on a gelfoam sponge showed that 5 min after stimulus the mRNA for PDGF-β and PDGF-β-receptor was increased, while pp60^{src} was activated and translocated to the actin cytoskeleton (Liu *et al*. 1995, 1996b).

There are many studies on growth regulation in pulmonary tumour cells, and an increasing literature on the roles of specific genes which control cell behaviour, in particular Hox genes and cellular oncogenes (Janssen *et al*. 1994; Minoo & King 1994; Shin *et al*. 1994; Hackett *et al*. 1996). Unfortunately in many of these reports, the specific cells where expression occurs is often not stated (or known), which means that firm conclusions are hard to reach about their involvement in stem cell biology and their relationship to carcinogenesis.

Ontogeny of pulmonary tumour cells

Tumour and normal lung epithelia could share cell lineages, but these are not yet satisfactorily proven. Several theories of lung tumour origins try to resolve the many phenotypes displayed by these neoplasms. Further details are to be found in recent reviews of the subject: (Baylin 1985; Barsky *et al*. 1994; Colby *et al*. 1995; Eimoto *et al*. 1985; Gatter *et al*. 1985; Marchevsky 1990b; Wright 1990; Mabry *et al*. 1991; Gatter & Dunhill 1992; Ratcliffe 1992). These range from the pluripotent stem cell hypothesis, where phenotypes are produced by differentiation (Müller & Fisseler-Eckhoff 1989), to the multiple primary hypothesis, a possibility inferred from analyses of aneuploidy in different foci of the same tumour mass (Barsky *et al*. 1994; Colby *et al*. 1995). It is a histological truism that many pulmonary tumours have at least partial phenotypes of several lung cells, often simultaneously, and many descriptions exist (van de Velde 1990; Dvorak & Monahan-Early 1992a, b).

Some tumours may indeed derive from a single stem cell type. For instance the Müllerian mixed cell tumour of the ovary has been suggested as a candidate (Kitagawa *et al*. 1996). Such tumours comprise an epithelial and mesenchymal component. Two cloned cell lines, OVAK I and II, were produced from one such tumour. OVAK I was non-tumourigenic in nude mice, while the other was. Upon TGF-β treatment, OVAK II tumour cells underwent

an epithelial to mesenchymal transition, at which time CEA expression fell and vimentin levels rose; two features of mesenchymal cells. It was concluded that the OVAK II cell line represented the single stem cell from which a mixed cell tumour could arise.

Multiple cell origins for tumours also have candidates. It is held that atypical adenomatous hyperplasia is a precursor lesion of bronchoalveolar carcinoma (BAC) (Kitamura *et al*. 1995, 1996), but that there is a spectrum of disease which is hard to subclassify. Nuclear size, hyperchromasia, atypia, p53 and CEA contents all rise with tumour stage, while mucous and ciliated cell numbers decline. The cells of origin for the BAC could only be given as either alveolar or bronchiolar.

The lineage in the gastrointestinal tract is now generally accepted as a monoclonal crypt which can continuously give rise to at least four cell types (stem, mucous, absorptive, neuroendocrine, and probably also Paneth cells) (Kirkland 1986, 1988; Potten & Loeffler 1990; Kirkland & Henderson 1994). This is the unitarian theory. The overall conclusions from much evidence are that intestinal differentiation in crypts is one-way, and that statistical chances exist for the development of each phenotype which depend on a cell's incoming signals and the subset of genes being transcribed (the so-called 'Gatter Conjecture') (Gatter *et al*. 1985; Wright 1990). Cloned (and subcloned) colorectal tumour cell lines which differentiate into four colonic crypt phenotypes (stem, absorptive, mucous and neuroendocrine) is good evidence for the clonal origin of crypts (Kirkland 1986, 1988, 1989; Kirkland & Henderson 1994), and many studies using chimaeras substantiate this (Potten & Loeffler 1990; Thompson *et al*. 1990; Paulus *et al*. 1992; Loeffler *et al*. 1993; Li *et al*. 1994). A parallel exists in leukaemias, where evidence for stem cell differentiation in a particular direction defines the tumour phenotype (Fialkow *et al*. 1987; Keinanen *et al*. 1988). On these grounds it may be reasonable to make a similar case for lung epithelia. Unfortunately, normal pulmonary cell lineages, traced *in utero*, adult life or after wounding, have not been shown conclusively to be matched by tumour cells, nor yet that each phenotype can be derived from a single cloned tumour cell (*in vitro* or by xenotransplantation). This may partly reflect the lack of a clearly defined functional unit in the lung, compared to the crypt. A further awkward difficulty is the differentiation of foetal lung epithelial cells, which appear in the reverse order of a supposed adult differentiation pathway (Baylin 1985; McDowell *et al*. 1993), yet the former pathway is supposed to exist in tumours too (van de Velde 1990). Indeed this may be a good argument for multiple lineages in pulmonary epithelia, where there is no functional equivalent of an intestinal crypt in which to define temporal and spatial cell kinetics. Only one pulmonary cell lineage could be inferred from the experiment using the re-inoculation of tracheas with Clara cells, for example, since this only gave rise to more Clara cells and ciliated cells. No evidence was found of tracheal basal cell or type II pneumocyte differentiation (nor neuroendocrine cells, though this was not sought) (Inayama *et al*. 1988, 1989; Nettesheim *et al*. 1990). This may be unfair to the Gatter Conjecture, since, with a strong influence of ECM and mesenchymal cells on epithelial gene expression and differentiation (Minoo & King 1994), it is to be admitted that the experiment did not fully recombine cells and their natural environments, so it could be argued that no deductions may be made about the inoculated cell's true potential. As noted before, the lineage potential of pure tracheal mucous cells needs to be defined in *in vivo* tracheal and bronchiolar settings, and to be compared with Clara cells, and also type II pneumocytes. These experiments will make a better judgment of lineages easier. It is no less important to test the ability for neuroendocrine cell differentiation from each of these supposed precursor cells.

The neuroendocrine cell is well studied in small cell lung carcinomas (SCLC) (Ratcliffe 1992; Cook *et al*. 1993), yet it is hardly detected in the cell renewal studies (see above) (McDowell *et al*. 1990; Nettesheim *et al*. 1990). The pluripotent nature of the 'indifferent' cell (McDowell *et al*. 1978; Müller & Fisseler-Eckhoff 1989) in human bronchial epithelium, and the 'transient endocrine' cell giving rise to an 'indifferent' cell (Ratcliffe 1992) are still essentially speculative lineages. Chimaeric lung experiments could be useful in this context; indeed such tissue may already exist from previous studies. Male/female chimaeras are a powerful tool, and DNA analyses of the temporal sequences of cell formation, patch sizes and clonality will help resolve these puzzles. An analysis of label-retaining cells could be useful in pulmonary lineages as in other tissues (Cotsarelis *et al*. 1990; Morris *et al*. 1990; Pavlovitch *et al*. 1991; Wei *et al*. 1993, 1995; Morris & Potten 1994) but no such studies exist at present. Micro-surgical tracheal or bronchial wounding combined with fluorescent cell lineage tracers may also be a useful experimental approach.

Conclusion

The evidence reviewed points to three potential lung epithelial cells with stem cell characteristics: the tracheal and bronchial basal cell, the bronchiolar Clara cell and the alveolar type II pneumocyte.

It remains to be seen whether the bronchial mucous

cell can repopulate the epithelium after injury, or by how much cell migration from the mucus glands onto the bronchial surface itself may augment this. The full differentiation potentials of the tracheobronchial indeterminate or indifferent cells are also lacking.

The cell lineage in the bronchus is probably: basal to secretory to ciliated, with a coincident fall in the likelihood of cell proliferation, most certainly in the latter cells. The bronchiolar lineage looks simpler and is probably: Clara cell to ciliated cell. Whether distal bronchial basal (or other) cells can produce proximal bronchiolar Clara cells is still unknown. It is possible that, *in extremis*, distal bronchiolar Clara cells will repopulate alveoli by providing a rescue lineage into type II pneumocytes. The more usual alveolar lineage is: type II pneumocyte to type I pneumocyte.

The position of the neuroendocrine cell in each of these lineages remains controversial, if not a conundrum. By their normal scarcity and their almost complete absence of cell cycle activity, it seems probable that they represent a numerically minor lineage derived from each pulmonary region. Candidate precursor cells are the indifferent/indeterminate cells in the tracheobronchial epithelium, and possibly Clara cells or type II pneumocytes. From EM studies of tumour cells possessing dual features of both NEC and either Clara or type II pneumocytes, these possibilities exist. The high incidence of NEC-like cells in many pulmonary tumours may represent cell lineage switching to favour their differentiation, possibly in a recapitulation of the foetal sequence. The signals for such development remain obscure, but could be auto- or paracrine. Future reports on these matters are awaited with interest.

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