Radioautographic study of human pulmonary tissues[1]

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The radioautograms of human lungs were studied after *in vitro* incubation with tritiated thymidine. Our materials consisted of the lungs of 12 normal foetuses (crown–rump length varied from 7·5 to 16·5 cm.) and of 11 adults (32 to 68 years of age). In the foetal lungs the uptake of tritiated thymidine was highest in the epithelium surrounding small tubular spaces, forerunners of future bronchial epithelium, and in the undifferentiated mesenchyme. The percentage of labelled cells was scarce in the alveolar wall cells of adults (0·05% to 0·65%), was unrelated to age, and appeared to be most frequent in phagocytic elements (so-called alveolar cells). There was an uptake of tritiated thymidine in scattered free alveolar macrophages and in a few interstitial cells. The media and adventitia of blood vessels did not show labelling in most cases. The labelling of epithelium of bronchioles and respiratory bronchioles varied from 0·20% to 0·70%. The findings of other investigators in the study of cell turnover in the lungs of laboratory animals are discussed and compared with the data obtained in the present study.

The kinetics of cellular proliferation in pulmonary tissues have been studied by several investigators in the last 15 years. These studies were carried out mainly in small laboratory animals (mice, rats, and guinea-pigs) using mitotic counting after colchicine arrest or radioautography with tritiated thymidine. Tritiated thymidine is incorporated almost exclusively into cell nuclei synthesizing deoxyribonucleic acid assumed to be in preparation for mitosis and, with high resolution radioautography, is used extensively in the study of cellular proliferation. Because of lack of data on cell proliferation in human pulmonary tissues, the present investigation was undertaken to study the uptake of tritiated thymidine *in vitro* in normal foetal and adult lungs with radioautography. Although the life span of different cell populations and the duration of phases of cell cycle cannot be determined by *in vitro* as by *in vivo* techniques, nevertheless it provides valuable information about the proliferative potentials of different cells. The data obtained can also be used as a base line in further investigations of cellular kinetics in pulmonary diseases.

MATERIALS AND METHODS

Pieces of lung tissue were obtained from 12 foetuses and 18 adults, though seven samples from adults were later discarded. The foetal lungs were obtained at hysterotomy from 12 normal foetuses measuring 7·5 to 16·5 cm. crown–rump. Immediately after removal of the conceptus, the lungs were dissected under sterile conditions, and slices were cut from different areas, each approximately 0·1 to 0·2 cm. thick and 0·3 to 0·4 cm. in diameter.

Slices of lung tissue were obtained from 18 adult patients at operation. In eight of the 18 cases incidental biopsies of the lung were obtained from patients undergoing non-pulmonary chest surgery, like a Vineburg procedure (translocation of the mammary artery into the myocardium), repair of diaphragmatic hernia or biopsy of mediastinal lymph nodes. One patient was undergoing pneumonectomy for carcinoma of the lung, and a biopsy was obtained from grossly normal areas before ligation of the pulmonary vessels. In nine of the 18 cases slices were obtained from grossly normal areas of the lung within a short time after lobectomy or pneumonectomy performed for carcinoma of the lung (8 cases) or for bronchiectasis (1 case). The tissue samples measured approximately 0·4 to 0·5 cm. in area and 0·2 cm. in thickness. All adult lung tissues were obtained between 9 a.m. and 3 p.m. The clinical and pathological data are listed in Tables I and II.

TABLE I

CROWN–RUMP LENGTH AND SEX OF FOETUS AND PERCENTAGE OF LABELLING OF DIFFERENT FOETAL PULMONARY TISSUES

Case	Sex and Crown–Rump Length (cm.)	2,000 Cells Counted in Each Case		1,000–2,000 Cells Counted in Each Case	
		Epithelium of Small Bronchi (%)	Undifferen-tiated Mesenchyme (%)	Epithelium of Large Bronchi (%)	Future Media and Adventitia of Blood Vessels (%)
1	M 7·5	15·4	9·5	—	—
2	F 8·0	16·8	10·4	6·8	8·2
3	M 8·2	13·1	10·1	6·1	7·4
4	M 10·5	13·7	10·8	6·3	7·8
5	M 11·5	10·1	4·1	—	—
6	M 12·0	11·6	7·3	5·3	7·4
7	F 12·3	12·4	6·7	5·9	6·9
8	M 12·5	9·1	7·2	5·4	7·2
9	M 13·0	7·7	4·2	3·4	4·6
10	M 13·2	9·6	7·3	5·0	3·6
11	F 14·5	6·9	5·2	4·0	6·3
12	F 16·5	6·4	6·8	3·9	4·9

Shortly after removal the tissue samples were incubated for two hours at 37° C. in 100–150 ml. Ringer's solution containing 1 microcurie tritiated thymidine per millilitre. A mixture of 95% oxygen and 5% CO_2 was bubbled through the Ringer's solution at a sufficient rate to keep the tissues in constant motion. After incubation the tissues were washed in three changes of Ringer's solution, fixed in 10% neutral formalin, dehydrated, and embedded in paraffin. Sections were cut at 5 μ to a depth of 50 μ. The slides were then deparaffinized, stained in eosin, rehydrated, and dipped in NTB2 Kodak emulsion. The emulsion-coated slides were exposed for six weeks in the ConRad/Joftes fluid emulsion radioautography system in CO_2 atmosphere and a relative humidity below 15%. After exposure they were developed for 2 minutes in Kodak D-19 developer, 15 seconds in Kodak SB5a stop bath, and kept for twice the clearing time (approximately 6–8 minutes) in Kodak acid fixer. All solutions were maintained at 18° C. throughout the process. The developed slides were washed for 1–2 hours, stained with haematoxylin, dehydrated, and mounted in balsam.

In the foetal lungs the percentage of labelled cells was determined by counting 4,000 to 8,000 cells in each case. These included 2,000 epithelial lining cells around small tubular spaces (the lumen measured 0·05–0·15 cm. in diameter) which represent the epithelium of future bronchi; 1,000 to 2,000 epithelial cells of larger bronchi (the lumen measured 0·2–0·7 cm. in diameter), 2,000 undifferentiated mesenchymal cells, and 1,000 to 2,000 cells in the future media and adventitia of blood vessels. The branches of larger bronchi were recognized by the characteristic longitudinal folds of the mucosa and the frequent presence of primitive cartilage around the wall. In two foetuses no larger bronchi or blood vessels with distinguishable walls were found.

In the adult lungs the labelled alveolar wall cells (cells lining the lumen of alveolar spaces), free alveolar macrophages, and interalveolar interstitial cells were counted in and among 4,000 alveolar spaces. The total number of nuclei lining 100 alveolar spaces was determined in each instance. From these data the number of alveolar wall cells, estimated to be present in 4,000 alveoli, was determined, and the percentage of labelled alveolar wall cells was calculated. The percentage of labelled cells was determined by counting 1,000 to 2,000 epithelial cells in the bronchioles (the wall smaller than 0·1 cm. in diameter) and respiratory bronchioles, and 2,000 cells in the media and adventitia of blood vessels. Only cells with five or more silver grains over the nucleus were considered to be labelled. The background labelling was low and random in all cases. Seven cases were omitted from this study because of the presence of inflammation in the slides examined. In all seven cases the tissue was obtained following pneumonectomy performed because of carcinoma of the lung.

RESULTS

The results are shown in Tables I and II.

TABLE II

AGE, SEX, CLINICAL DIAGNOSIS OF LUNG DISEASE, AND NUMBER AND PERCENTAGE OF LABELLING OF DIFFERENT TYPES OF CELLS IN ADULT LUNGS

Case	Sex and Age (yrs)	Clinical Diagnosis	No. and % Labelled Alveolar Wall Cells in 4,000 Alveoli	No. of Labelled Free Alveolar Macrophages in 4,000 Alveoli	No. of Labelled Interstitial Cells Among 4,000 Alveoli	% of Labelled Bronchiolar Epithelium (1,000–2,000 Cells Counted)	% of Labelled Cells in Wall of Blood Vessels (2,000 Cells Counted)
1	M 32	—	35 (0·19%)	24	7	0·70	0·3
2	F 33	Bronchiectasis	66 (0·34%)	48	4	0·20	0·15
3	M 35 Vessels tied	—	17 (0·08%)	36	4	0·15	0
4	M 54	—	106 (0·55%)	49	6	0·40	0
5	M 54	—	12 (0·08%)	11	7	0·50	0
6	M 55	—	48 (0·25%)	31	8	0·30	0
7	M 56	—	20 (0·10%)	15	8	0·45	0
8	M 56	—	10 (0·05%)	14	3	0·40	0
9	F 58 Vessels tied	Carcinoma of lung	113 (0·65%)	66	4	—	0
10	F 66	—	17 (0·09%)	29	4	0·60	0·05
11	M 68	Carcinoma of lung	14 (0·08%)	33	8	0·60	0·05

The pulmonary vessels were ligated in only two cases before tissues were obtained.

In the foetal lungs the percentage of labelled cells was highest in the epithelium surrounding small tubular spaces (7·7% to 16·8%) (Fig. 1). The majority of investigators, including us, believe that these structures represent the epithelium of future bronchi and not air spaces. The labelling of un-differentiated mesenchymal cells (5·2% to 10·4%) was lower than in the epithelium of small bronchi, but higher than in cells forming the wall of blood vessels (3·6% to 8·2%) (Fig. 2) or in the epithelium of larger bronchi (3·9% to 6·8%). In our material the uptake of tritiated thymidine was lowest in the epithelium of large bronchi among all types of cells examined.

The labelling of cells was generally higher in the younger foetuses, and seemed to decrease with gestational age. However, this was less conspicuous in the mesenchyme than in the bronchial epith-elium or in cells forming the blood vessel walls.

In adult lungs the number of labelled alveolar wall cells was small (0·05% to 0·65%) (Figs 3 and 4) and appeared to be unrelated to age, carcinoma, or inflammation in distant areas of the lung, or to short ischaemia due to ligation of pulmonary vessels. In the alveolar wall most of the labelling appeared to be present in phagocytic cells (so-called alveolar cells). The percentage of labelling was not determined in different types of alveolar wall cells because with the techniques employed it was not possible to identify each of them accurately. A number of free macrophages in the alveolar spaces had nuclear labelling (Fig. 5). The number of labelled interalveolar cells was small. In seven of 11 cases the media and adventitia of blood vessels did not show any uptake of tritiated thymidine. The range of labelling was small in the epithelium of bronchioles and respiratory bron-chioles (0·20% to 0·70%) (Fig. 6).

DISCUSSION

The primordia of lungs develop from the anterior wall of the foregut epithelium. The epithelial
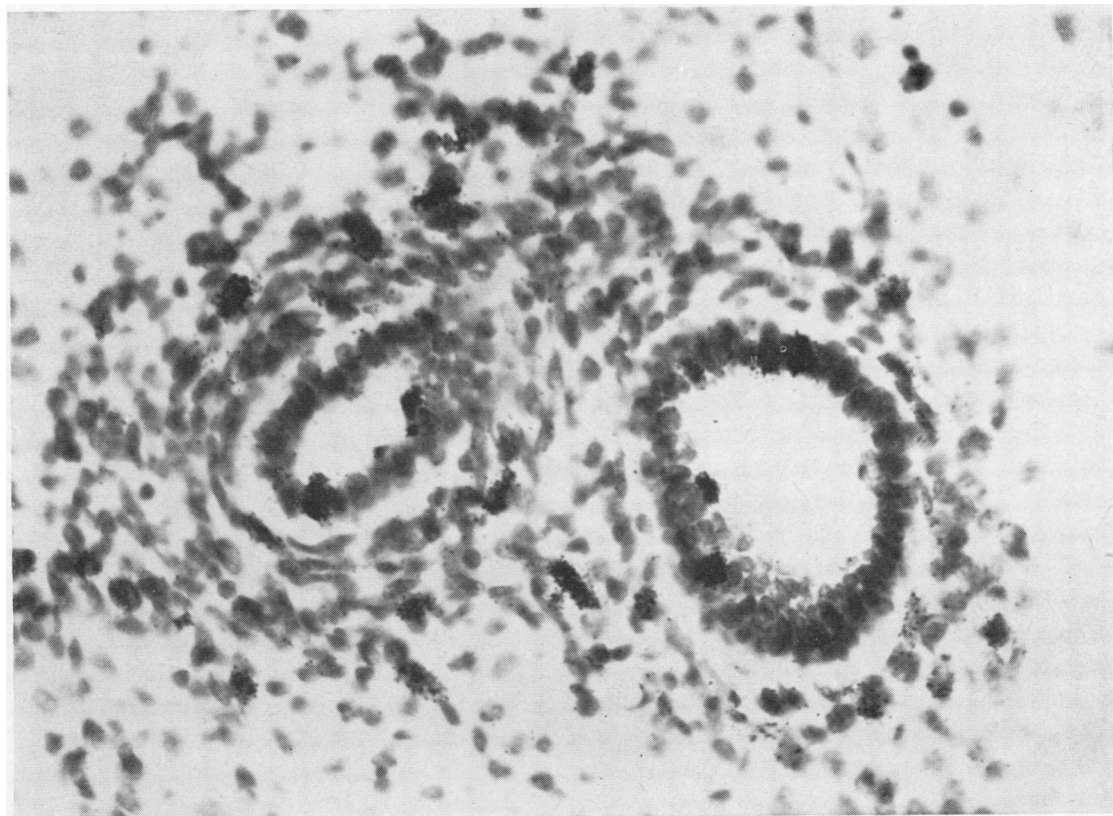

FIG. 1. *Uptake of tritiated thymidine in the epithelium surrounding small tubular spaces (forerunners of future bronchi) and in undifferentiated mesenchymal cells. (Crown–rump length of foetus 8.2 cm.) H. and E. ×425.*
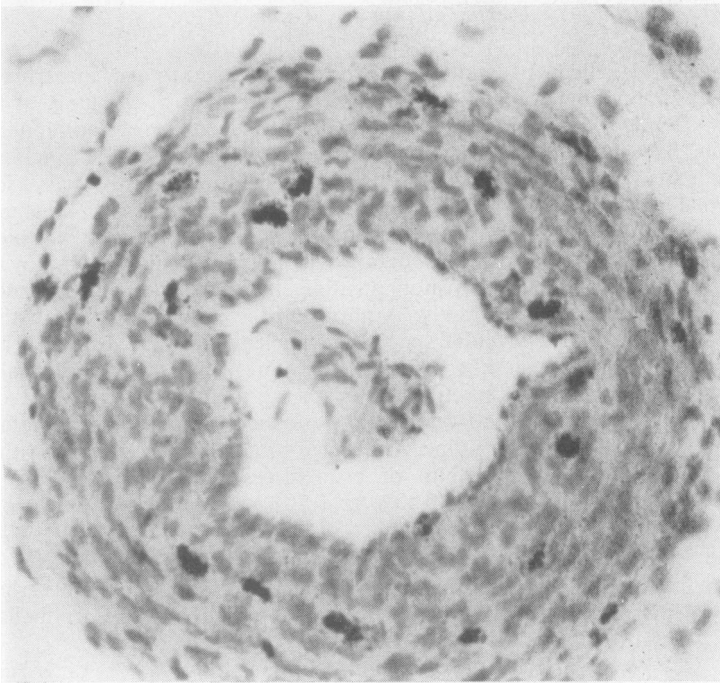
FIG. 2 (left). Uptake of tritiated thymidine in the wall of a foetal pulmonary blood vessel. (Crown–rump length of foetus 10.5 cm.) H. and E. ×400.
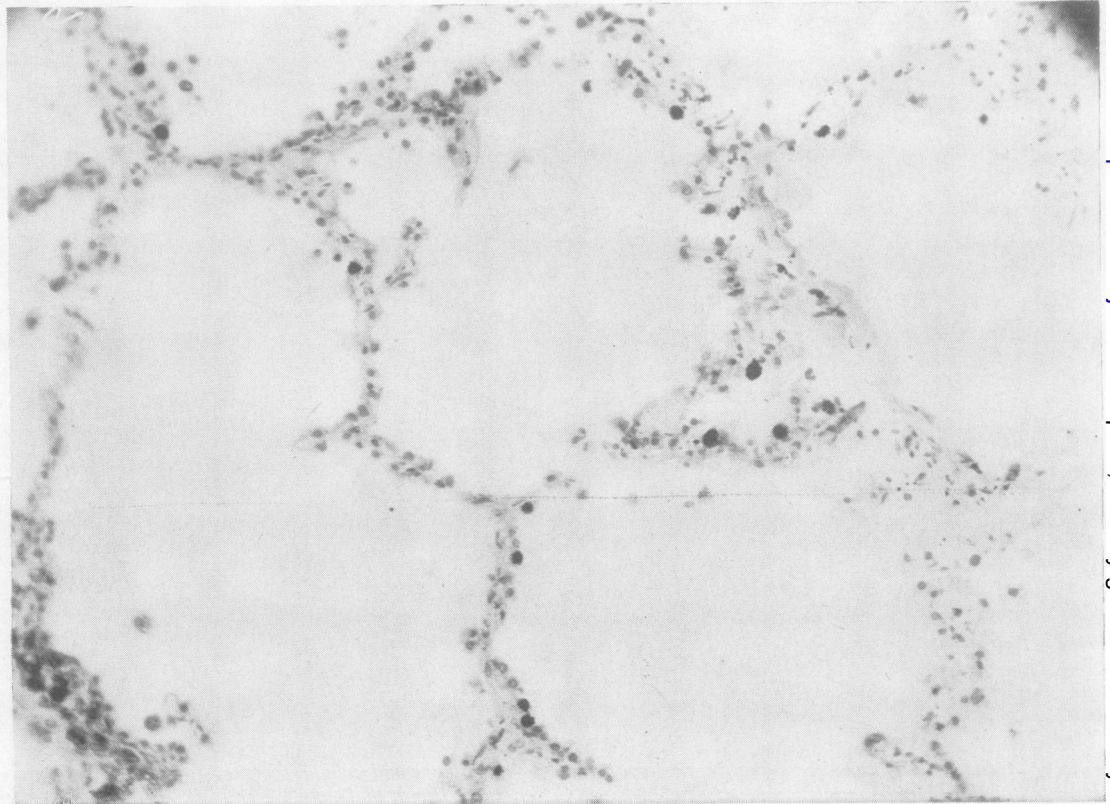
FIG. 3 (below). Labelled alveolar wall cells in the lung of a 58-year-old woman. Pneumonectomy was performed because of carcinoma of the lung. H. and E. ×210.

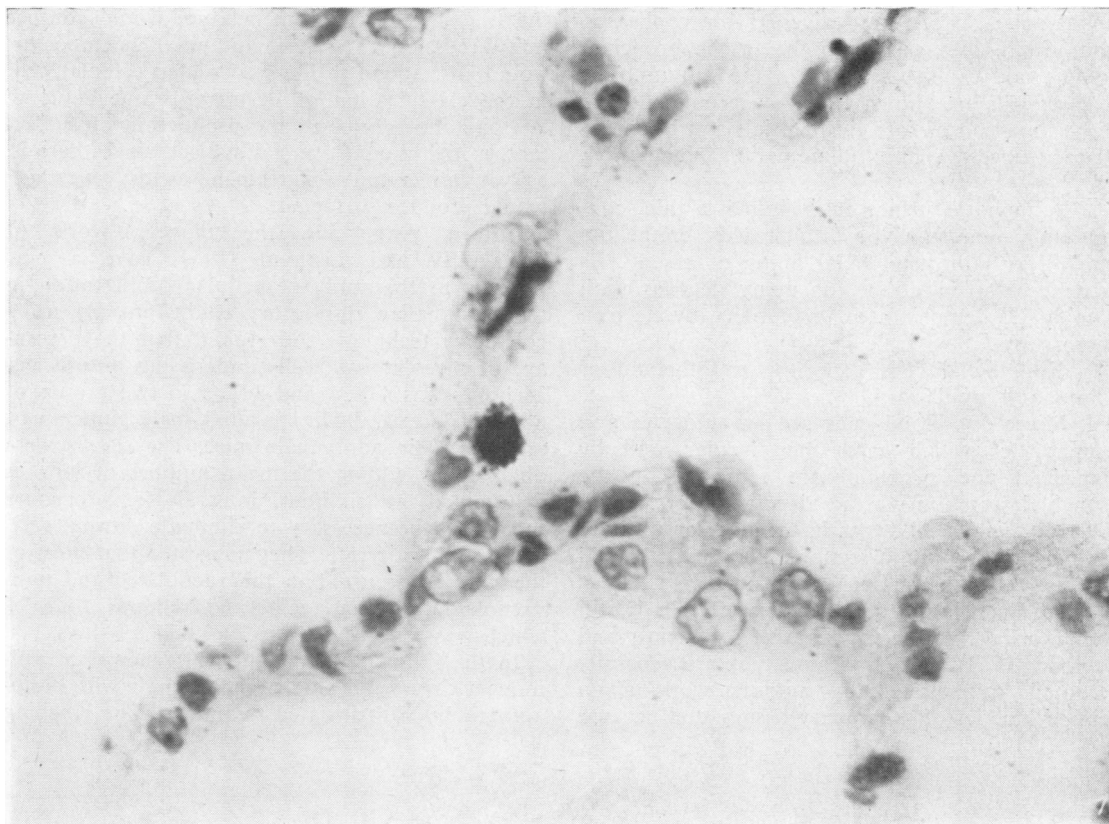FIG. 4. *Labelled alveolar wall cell in the lung of a 32-year-old man. H. and E.* ×850.

tubes, representing the future main bronchi, give rise to a rapidly growing and branching duct system, forerunner of the epithelium of future bronchi, bronchioles, and respiratory bronchioles. This embryonic epithelium is columnar and cuboidal, and is surrounded by undifferentiated mesenchyme from which the wall of the bronchial tree, including the cartilage, arises. The bronchial tree, from the main bronchi to the respiratory bronchioles, seems to arise during the first half of foetal development. Most investigators think that the respiratory epithelium (alveolar ducts and alveoli) starts to appear in embryos measuring 14–15 cm. in length. During subsequent growth the alveolar spaces rapidly increase in number and size, and become increasingly vascularized by proliferating capillaries. There is a conspicuous reduction of foetal mesenchyme during this time with almost complete disappearance before term (Cooper, 1938 ; Ham, 1965 ; Hamilton, Boyd, and Mossman, 1962 ; Norris, Kochenderfer, and Tyson, 1941).

Our results correspond well with previous classical histological studies. The high rate of DNA synthesis, assumed to be in preparation for mitosis, is found in the bronchial epithelium during a period when the bronchial passages are known to arise. It is interesting that labelling is the highest in the small epithelial tubules and surrounding mesenchyme since organ culture studies also demonstrated that the foetal human lung expands by extensive bifurcation and budding of the terminal part of the bronchial tree and surrounding mesenchyme (Glucksmann, 1964).

Since it was realized that there are different kinds of cells in the wall of adult mammalian alveoli, some controversy has arisen about their nomenclature. Bertalanffy and Leblond divided the alveolar wall cells into the following categories (Bertalanffy and Leblond, 1955 ; Bertalanffy, 1965):

1. Alveolar cells (vacuolated and nonvacuolated types) are considered to be the most conspicuous cells in the alveolar wall. They desquamate into

the alveolar spaces (free alveolar macrophages), from where they pass into the tracheobronchial exudate and can be detected in the sputum. The alveolar cells are known to have a prominent role in the ingestion of foreign materials and in pathological processes like inflammation, congestion, etc.

2. Pulmonary surface epithelium is a thin, continuous, nonphagocytic, nucleated epithelium which forms the superficial lining of alveoli. Its existence was in doubt for many decades until Low (1953) demonstrated its presence by electron microscopy.

3. Endothelial cells form the endothelium of pulmonary capillaries.

4. Migratory blood cells are granulocytes and lymphocytes which enter the alveolar wall by diapedesis and probably are removed by the bronchial secretion via the alveolar spaces.

These various cell types in the alveolar wall are distinguishable only in sections fixed in Orth fluid and stained with Masson's trichrome.

The nomenclature of alveolar wall cells is still controversial. We use the terms of Bertalanffy and Leblond (1953) mainly because they are generally accepted and quoted by authors working on cellular kinetics of pulmonary tissues, and because the use of different nomenclature would confuse our discussion. The term 'granular pneumocyte' would correspond to the 'vacuolated alveolar cell' in the classification of Bertalanffy and Leblond. We have not discussed the production of surfactant, a highly controversial subject, since there is, as yet, no common agreement as to which cell would produce surfactant.

Fifteen years ago the pioneer work of Bertalanffy and Leblond (1953) gave a new impetus to the study of cellular proliferation in the mammalian respiratory epithelium. By using colchicine technique they found that the percenage of alveolar wall cells undergoing mitosis per 24 hours was 4·06% and 3·57% in two groups of adult male rats, 3·49% in adult male guinea-pigs, and 4·52% in adult male mice. The results were obtained by adding the mean number of mitoses determined at six-hour intervals—a procedure thought to be necessary to eliminate diurnal variation of mitotic rate. They suggested that most of the mitoses occurred in the vacuolated and non-vacuolated alveolar cells, the renewal time of which was estimated to be 29·4 and 8·1 days.

In the following years the cell renewal of pulmonary tissues was studied increasingly with radioautography with tritiated thymidine. The labelling
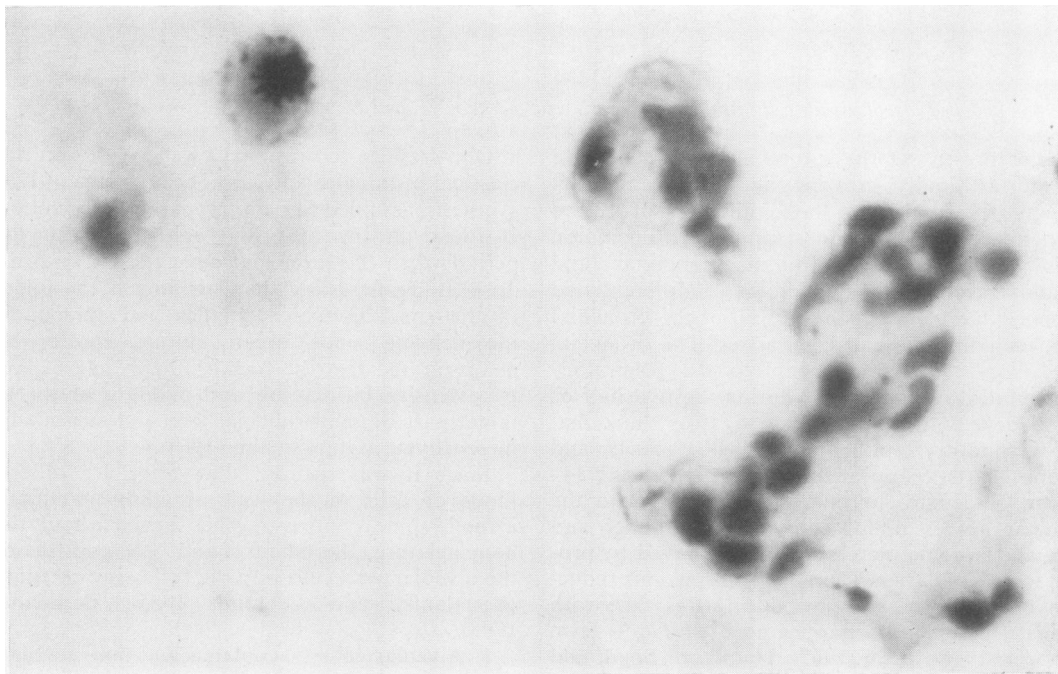

FIG. 5.   *Labelled free alveolar macrophage in the lung of a 35-year-old man.   H. and E. ×1,000.*
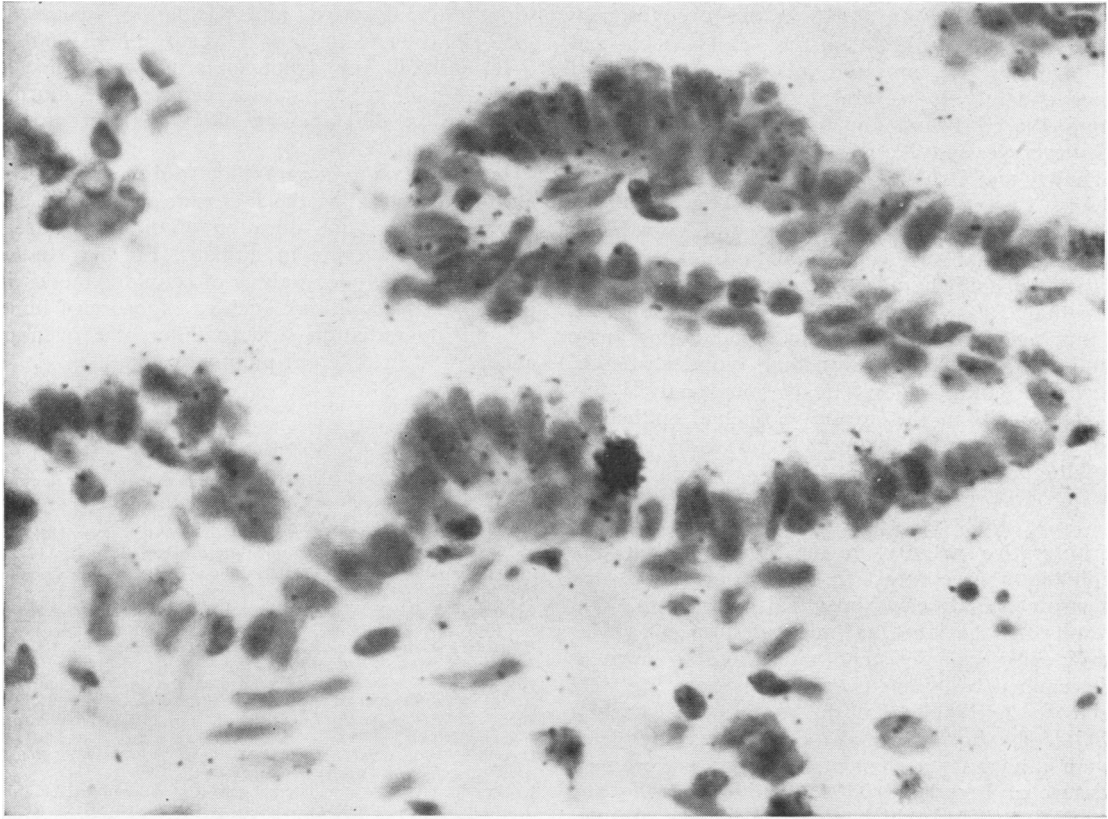
FIG. 6. *Uptake of tritiated thymidine in the bronchiolar epithelium of a 32-year-old man. H. and E.* ×850.

index of alveolar wall cells at any single time was found to be 1·8% by Schultze and Oehlert (1960) (in adult rats and mice), 0·4% by Edwards and Klein (1961) (in adult mice), and 0·25% to 1·0% by Shorter and Titus (1962) (in adult mice). In the experiments of Shorter, Titus, and Divertie (1964) and Spencer and Shorter (1962) the percentage of labelled alveolar wall cells varied from 1·2% to 2·1% in rats and from 0·4% to 5·0% in mice, but it decreased considerably after one week and was not seen in animals sacrificed 3 to 5 weeks after injection. These results suggest that two populations of alveolar wall cells (macrophages) exist, one with a turnover time of 7 days, the other with a turnover time of 14 to 21 days (rats) and 35 days (mice) (Shorter, Divertie, and Titus, 1964; Shorter, Titus, and Divertie, 1964, 1966). Simnett and Heppelston (1966) found a sex, strain, and age difference in the mitotic index of adult mice respiratory epithelium, but these differences were either absent or less striking in the radio-

autographic study of the same group of mice. Sex differences in the mitotic index of alveolar wall cells disappeared after 3 days of *in vitro* culture, suggesting that hormonal factors might be responsible which degrade in culture (Simnett and Heppleston, 1967a, b). In the experiments of Simnett and Heppleston (1966) a wide range of standard deviations was found; however, the mean labelling index of alveolar wall cells varied from 0·17% to 0·56% in different strains of adult mice.

The labelling and mitotic indices of alveolar wall cells were found to be higher in newborn than in adult animals (Kury, Craig, and Carter, 1967; Simnett and Heppleston, 1967a, b).

Although the emphasis in most investigations was on cell renewal of alveolar wall cells, the epithelium of the bronchial tree was also studied. The life cycle of bronchial epithelium is of critical importance to studies of bronchial carcinogenesis. The range of uptake of tritiated thymidine *in vivo*

varied from 0·4% to 1·9% in the large bronchi and from 0·2% to 1·0% in the small bronchi and bronchioles of rats and mice, as determined independently by several investigators (Blenkinsopp, 1967; Edwards and Klein, 1961; Meyer zum Gottesberge and Koburg, 1963; Koburg, 1962; Schultze and Oehlert, 1960; Shorter, Divertie, and Titus, 1964; Shorter, Titus and Divertie, 1964, 1966). The findings of Shorter and co-workers suggested that two cell populations exist in the bronchial epithelium of mice with turnover times less than 2 days or between 5 and 7 days (Shorter, Titus, and Divertie, 1964). In a radioautographic study of 62 human bronchial biopsies, Lesch, Schiessle, and Oehlert (1964) found only a few labelled cells in the normal bronchial epithelium in contrast to epithelium involved by chronic inflammation.

In our present study the number of labelled alveolar wall cells was small, indicating a low proliferative activity in the human respiratory epithelium in contrast to haematopoietic tissues, gastrointestinal or exocervical epithelium. Our results for the labelling index of human alveolar wall cells are consistent with those in mice as determined by Simnett and Heppleston. We were able to determine that the majority of alveolar wall cells showing uptake of tritiated thymidine were in phagocytic elements (alveolar cells, nomenclature of Bertalanffy (1965) and Bertalanffy and Leblond (1955)) which give rise to free alveolar macrophages. This finding is not surprising knowing the constant need and role of these cells in the defence processes of the lung. Our finding of uptake of tritiated thymidine in free alveolar macrophages, presumably in preparation for mitosis, is also of interest. Simnett and Heppleston (1966) observed mitotic figures in free alveolar macrophages in normal adult mice lungs by the colchicine technique. Mitotic figures are not infrequently seen in macrophages in the early stage of alveolar inflammation using the classical histological technique. It is only unfortunate that in previous radio-autographic studies of normal animals, free alveolar macrophages were not studied. This is possibly due to the fact that sometimes it is difficult to differentiate dust particles from silver grains. We omitted from our study cells in which differentiation was difficult.

The percentage of labelled human bronchiolar epithelium was small and was completely in the range of that of mice and rats as determined *in vivo* by several independent investigators (Blenkin-

sopp, 1967; Edwards and Klein, 1961; Koburg, 1962; Shorter, Titus, and Divertie, 1964, 1966).

Because all our adult material was collected from 9 a.m. to 3 p.m. we believe that diurnal variation in the labelling index did not greatly influence our results.

In vivo radioautographic studies would be needed to determine the life span of different cell populations and the duration of different phases of the mitotic cycle in humans. Further studies should also include analysis of cellular kinetics in pathological conditions, such as exposure of lungs to ionizing radiation, and to different exogenous agents, and in different pulmonary diseases.

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