The Biochemistry of Epithelia: Enzyme Levels in Bronchial and Tracheal Epithelia of Smokers and Non-Smokers and in Bronchial Carcinoma

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Spencer (1958) developed a method for the removal of bronchial epithelium from human autopsy specimens in a form suitable for the estimation of various biochemical entities and allowing a strict cytological basis for their expression. Investigation of the epithelial-cell suspensions so prepared established conditions for the assay of several hydrolytic enzymes and showed the validity of the assays in autopsy material removed up to 6 hr. *post mortem* (Spencer, 1959). The material was stored at -20° with no noticeable decrease in the enzyme activity after 6 months.

The present study was designed to investigate the effect of smoking on the biochemistry of bronchial epithelium. The rationale of the study was to measure various enzymes and substances in suspensions of human bronchial epithelium removed at autopsy and to correlate the routine assays with smoking habits and clinical records in an attempt to detect precancerous 'biochemical lesions' which could be associated with the inhalation of tobacco smoke. Various histological precancerous lesions have already been reported to be more numerous in the tracheobronchial tract of smokers than in non-smokers (Auerbach *et al.* 1957*a, b*; Chang, 1957; Ide, Suntzeff & Cowdry, 1959).

The occurrence of carcinoma of the trachea is very low compared with that of the bronchus and no significant rise in incidence of tracheal carcinoma has been observed during the last few years to parallel the increase in bronchial carcinoma (cf. Griswold, Wilder, Cutler & Pollack, 1955), which the majority of epidemiological studies have associated with the smoking habit (for a review see Davies, 1956). Comparison of the bronchial epithelium with the tracheal epithelium from the same subject was therefore made to distinguish between carcinogenic and non-specific effects of tobacco smoke. To avoid confusion between changes due solely to variations in cell population of the epithelia and specific precancerous changes, the biochemical assays were related to the cytological make-up of the epithelial-cell suspensions.

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Further aid to the recognition of cancerous and pre-cancerous changes was sought by comparison of the enzyme levels in tracheobronchial epithelia with those of human-lung squamous-cell carcinomas removed at surgery and human bronchial carcinoma transplants in hamster cheek-pouch. To increase the possibilities of detecting any pre-cancerous changes, epithelial-cell samples were removed from the right main-stem bronchus at a position where the incidence of squamous-cell carcinoma is greatest, and the cases studied were restricted to males over the age of 40 years, the age group in which bronchial carcinoma is most frequent. A number of enzymes, selected for reasons stated by Spencer (1959), have now been studied in this material.

EXPERIMENTAL

Autopsy material. The autopsy specimens considered for analysis were restricted to those from white and coloured American males over the age of 40 years. Within this broad specification considerable care was exercised in the selection of specimens. Contra-indications included the lapse of more than 6 hr. between death and cooling of the removed epithelial-cell suspension to -20° ; presence of purulent mucus; terminal pneumonia, bronchitis or other respiratory disease; aspiration of gastric contents; extensive haemorrhage; inanition. The specimens examined totalled 87 and only 22 proved to be suitable for the present investigation. Two further cases with terminal pneumonia were also investigated. Of the cases analysed, nine were non-smokers, whereas the others had smoked cigarettes to various degrees and for various lengths of time. In view of the final findings no attempt has been made to classify the smokers into different groups.

Preparation of samples. The epithelium from 2 cm.² of the right main-stem bronchus approx. 2 cm. below the bifurcation was suspended in 1.2 ml. of ice-cold iso-osmotic sucrose (0.25M) containing 0.01M-ethylenediaminetetraacetate (sodium salt) at pH 7.4, by the brush technique of Spencer (1958). The epithelial-cell suspension was immediately distributed in 10, 20 and 40 μ l. portions among a number of small tubes, the design of which was dictated by the different enzymes and substances to be examined (Spencer, 1959). The tubes were frozen immediately and stored at -20° until required for assay. Other portions were retained for total and differential cell counts in duplicate. The denuded areas of the bronchus were fixed in Bouin fluid and sectioned, and sections were stained with

haematoxylin and eosin and examined microscopically to check the extent of removal of the epithelium (Spencer, 1958). Removal was complete in all cases. The epithelium from a 2 cm.² area of the trachea 3–5 cm. above the caryna was similarly treated. Portions of the bronchus and trachea contiguous with those removed for biochemical analysis were examined in a cytological study which has been reported elsewhere (Ide *et al.* 1959).

Human bronchial (squamous-cell) carcinoma samples were obtained within 0.5 hr. after surgical removal. Portions about 1 cm.³, which were macroscopically non-necrotic, were placed on a microscope slide and coarsely minced with a scalpel. Pressure on the cut tissue with the flat blade of the scalpel resulted in an exudation of the loosely packed carcinoma cells. The exudate was suspended in 2 ml. of ice-cold 0.25 M-sucrose, mixed by five passages through the orifice of a pipette and allowed to stand for about 2 min. at 0°. The material still in suspension was removed with a pipette, leaving connective tissue at the bottom of the tube. The carcinoma-cell suspension was diluted with cold 0.25 M-sucrose to a concentration of about 6×10^6 cells/ml., which was suitable for the enzyme estimations. The diluted suspension was distributed amongst a number of small tubes as for the epithelial-cell suspensions and then frozen and stored at -20°. Further portions were retained for total and differential cell counts. These preparations showed negligible contamination with connective-tissue cells and leucocyte contamination was below 5%.

Human bronchial carcinoma transplants in the cheek-pouch of cortisone-treated hamsters (Sirota & Wishnow, 1958) were removed 9 days after transplantation and treated in a similar fashion to the surgically removed specimens. The transplanted tumours were highly vascularized and careful dissection of the material was necessary before mincing to restrict the contamination of the final cell suspension by blood cells to below 5%.

Cytoplasmic volumes. Smears of the cell suspensions were stained with Alcian Blue and Neutral Red (Spencer, 1958) and the areas of each of 25 goblet, ciliated, intermediate and basal cells and their respective nuclei, selected at random, were measured with a camera lucida and a planimeter. The cells and nuclei were classified as spheres, prolate spheroids, cylinders or cones, depending on their sectional shape, and their volumes were calculated by multiplying their diameters by the factors 0.67 (spheres), 0.67 ($\times$ the smaller diameter, prolate spheroids), 0.79 (cylinders) and 0.52 ($\times$ the diameter across the top, cones). After subtraction of the volumes of the individual nuclei the volume of the cytoplasm of each cell and then the average cytoplasmic volume of each type of cell was calculated. From the differential cell count it was possible to calculate the overall average cytoplasmic volume of the cells of the suspension. The final value arrived at was considered only a rough approximation since the cells were never perfect geometrical shapes and since no allowance could be made for the flattening of the cells on the slide or for differential shrinkage between individual cells or even between cytoplasm and nuclei. A further source of error was the space in the theca of the goblet cells, but a partial correction was made by measuring only the volumes of empty goblet cells in which the theca is occupying a minimal volume. With Alcian Blue the mucus was stained blue and full and empty goblet cells could be easily distinguished.

Assay methods. Acid phosphatase, β -glucuronidase, arylsulphatases A and B and arylsulphatase C were assayed under the conditions previously found to be optimum for these enzymes in human bronchial epithelium (Spencer, 1959). Lactic and malic dehydrogenases were assayed by the method of Robins, Roberts, Eydt, Lowry & Smith (1956) in diluted 10 μ l. samples of epithelial suspensions. Protein nitrogen was measured in 10 μ l. samples by an adaptation (Spencer, 1959) of the method of Nyvar & Glick (1954).

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were measured in samples (20 μ l.) by an adaptation of the ultramicro method of Lowry, Roberts, Leiner, Wu & Farr (1954) and incorporating a separation of RNA and DNA by differential alkaline hydrolysis (Schmidt & Thannhauser, 1945). To a portion (20 μ l.) of the epithelial-cell suspension contained in a tapered tube (50 mm. long, 4.5 mm. internal diam.), cooled to 0°, was added 20 μ l. of cold 10% (w/v) trichloroacetic acid. After mixing by holding the tube against a flattened nail rotating at about 1500 rev./min. the tube was allowed to stand for 15 min. at 0°, vibrated again and then centrifuged at 0°. The supernatant was withdrawn with a fine-tipped pipette and 80 μ l. of cold ethanol containing 0.1 M-potassium acetate was immediately added. The tube was again vibrated, centrifuged and the supernatant was withdrawn. The precipitate was further successively washed on the centrifuge with 80 μ l. of ethanol and 80 μ l. of ethanol-ether (1:1, v/v), and then dried *in vacuo* for 1 hr. To the dried precipitate was added 20 μ l. of 0.2 N-KOH, the tube was vibrated, capped with Parafilm and incubated for 16–20 hr. at 38°. The sample was then cooled to 0° and 36 μ l. of 5% (w/v) perchloric acid was added. The tube was vibrated, centrifuged at 0° and the supernatant was transferred to a round-bottomed tube 50 mm. long, 4.5 mm. internal diam. The precipitate was washed on the centrifuge with a further 20 μ l. of 5% perchloric acid and the supernatants were combined (RNA sample). To the precipitate in the original tube was added 30 μ l. of 5% perchloric acid, the precipitate was suspended by vibration, the tube was capped tightly with a rubber stopper and heated at 75° for 1 hr. After cooling, the tube was centrifuged and the supernatant was transferred to a round-bottomed tube. The precipitate was washed on the centrifuge with 20 μ l. of 5% perchloric acid and the supernatants were combined (DNA sample). The phosphate contents of the RNA and DNA samples were then measured by the method of Lowry *et al.* (1954), with 10 μ l. of ashing mixture and 100 μ l. of the colour reagent. The estimation was carried out in triplicate together with triplicate water blanks, which were taken through the whole procedure. At a later stage it was found more convenient to use a 40 μ l. sample of the cell suspension for nucleic acid determinations. All volumes throughout the procedure were then doubled.

RESULTS

Acid phosphatase. The acid-phosphatase activity per cell varied in the different epithelial samples, the highest value being approximately five times that of the lowest. A similar widespread divergence was shown when the activities were calculated on the basis of the DNA contents of the cell suspen-

sions or on their protein-nitrogen contents. There was no significant correlation between enzyme activity and the number of goblet, ciliated or basal cells in the sample, but a rough correlation was seen when the acid-phosphatase activities were plotted against the ratio columnar (ciliated, goblet and intermediate): basal cells (Fig. 1). In normal human bronchial epithelia this cell ratio is approximately 1:1 (Spencer, 1958), and ratios of the order of 0.5:1 indicate a considerable degree of basal-cell hyperplasia. Ratios greater than 1:1 indicate an increased number of goblet and ciliated cells and also that these columnar cells are generally enlarged (cf. Ide *et al.* 1959). Since the columnar cells are much larger than basal cells the columnar:basal cell ratio is a rough guide to the average size of the cells in the different cell suspensions, and this suggested that the acid-phosphatase activities of the epithelial-cell suspensions might be a reflexion of the relative sizes of the cells. The nuclei of the basal and columnar cells are of similar size and the difference between the two types of cell lies in the large cytoplasmic volume of the latter type. RNA/DNA ratios are generally accepted to be a useful measure of cytoplasmic:nuclear ratios and, as shown below, there was good correlation between the RNA content and the cytoplasmic volume of the epithelial-cell suspensions. Accordingly the

acid-phosphatase activities were plotted against the average RNA content/cell.

A considerable degree of correlation was observed (Fig. 1). Most enzyme values lay within one standard error (drawn to the *Y* axis) of the calculated regression line, whether the activities were those of bronchial or tracheal epithelia or whether the epithelia were derived from smokers or non-smokers. The regression line passed through zero and had a correlation coefficient of 0.94. The acid-phosphatase activities of the five cell suspensions of surgically removed lung carcinomas and the two values for the human bronchial carcinoma transplants in hamster cheek-pouch were reasonable fits to the regression line (Fig. 1).

Other enzymes. Findings almost identical with those for acid phosphatase were made with β -glucuronidase, arylsulphatases A plus B, arylsulphatase C, lactic dehydrogenase and malic dehydrogenase (Figs. 2-6), although in no case was the correlation coefficient as high as for acid phosphatase. The enzyme activities of the human bronchial carcinomas and transplants in hamster were reasonable fits to the respective regression lines. Only one exception was noted; the arylsulphatase C values for bronchial carcinoma transplants in hamster were about five to seven times as high as was expected from their RNA content (Fig. 4). With lactic and malic dehydrogenases the

Fig. 1. Acid-phosphatase activity of epithelial-cell suspensions plotted against ribonucleic acid content. The regression line (correlation coefficient 0.94) is drawn as a continuous line and broken lines are drawn at a distance equal to one standard error of the estimate (*Y* axis). □, Bronchus, non-smokers; ■, bronchus, smokers; ○, trachea, non-smokers; ●, trachea, smokers; ▲, bronchial carcinoma, surgical specimens; △, bronchial carcinoma, transplants in hamster cheek-pouch. The average columnar/basal cell ratio is stated with the range in parentheses.

Fig. 2. β -Glucuronidase activity of epithelial-cell suspensions plotted against ribonucleic acid content. Details were as given in Fig. 1. The correlation coefficient of the regression line is 0.76.

slopes of the regression lines were shallow and did not pass within one standard error of the origin. The slope of the regression line for lactic dehydrogenase (0.41) had a standard error of 0.111 and was significantly different from the horizontal ($P < 0.01$).

Ribonucleic acid content and cytoplasmic volume. Although a small amount of RNA is found in nuclei of mammalian cells (Davidson, 1950), the major portion is known to be associated with the endoplasmic reticulum of the cytoplasm. Fig. 7 shows that in the selected samples of bronchial

epithelial-cell suspensions there was a reasonable correlation between the average cytoplasmic volume of the cells and their RNA content. This suggests that the enlarged size of the columnar cells, seen in various circumstances, particularly in the epithelia of mild smokers (Ide *et al.* 1959), is due to an increase in the amount of functioning cytoplasm rather than to oedema or other non-specific changes.

Fig. 3. Arylsulphatase A plus B activity of epithelial-cell suspensions plotted against ribonucleic acid content. Details were as given in Fig. 1. The correlation coefficient of the regression line is 0.84.

Fig. 4. Arylsulphatase C activity of epithelial-cell suspensions plotted against ribonucleic acid content. Details were as given in Fig. 1. The correlation coefficient of the regression line is 0.80.

Fig. 5. Lactic-dehydrogenase activity of epithelial-cell suspensions plotted against ribonucleic acid content. Details were as given in Fig. 1. The correlation coefficient of the regression line is 0.54.

Fig. 6. Malic-dehydrogenase activity of epithelial-cell suspensions plotted against ribonucleic acid content. Details were as given in Fig. 1. The correlation coefficient of the regression line is 0.76.

Deoxyribonucleic acid. Leuchtenberger, Leuchtenberger & Doolin (1958), using ultraviolet microscopy, have measured differences between the DNA values of individual normal mouse bronchial epithelial cells and metaplastic epithelia induced by exposure to tobacco smoke. In the present investigation the DNA modal values in $\mu\mu\text{g.}$ of phosphorus/cell were: bronchus, non-smokers, 0.65; bronchus, smokers, 0.72; trachea, non-smokers, 0.71; trachea, smokers, 0.72. Statistically these values were the same.

DISCUSSION

Since RNA is almost exclusively localized in the cytoplasm of cells it can be reasoned that when the RNA/enzyme-activity regression line (Figs. 1-6) is extrapolated to zero RNA, the indicated enzyme activity will represent that due to the cell nuclei (Fig. 7). With the enzymes acid phosphatase, β -glucuronidase, arylsulphatases A plus B and arylsulphatase C the regression lines all passed within one standard error of the origin of the axes (Figs. 1-4), and the nuclear activity of these enzymes can therefore be considered to be negligible. The cytoplasmic localization of these enzymes in human tracheobronchial epithelial cells is in accord with the results obtained for the same enzymes in rat-liver cells by the technique of differential centrifuging of cell homogenates. Although a good correlation between RNA content of the cells and enzymes which are localized in the endoplasmic reticulum might have been expected, a similar high degree of correlation was seen with acid phosphatase and arylsulphatases A plus B, which are found in the lysosomes (Viala & Gianetto, 1955; Dodgson, Spencer & Thomas, 1954), and β -glucuronidase, which is of mixed mitochondria-lysosome localization (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). The correlation of RNA content with the activities of these

various enzymes may indicate some direct relationship, or, both the RNA content and the enzyme activities may simply be reflexions of the variation in cytoplasmic size of the cell (Fig. 7). In either case the cells can be considered to possess a remarkable ability to maintain a cytoplasm of constant composition as far as the components examined are concerned.

At least two interpretations are possible in those cases (lactic and malic dehydrogenases, Figs. 5 and 6) where the RNA/enzyme-regression line cuts the abscissa at a point more than one standard error away from zero enzyme activity. The first is that the activity indicated on the abscissa is that of the nuclei of the cells. The second interpretation invokes the presence of two sources of the enzyme in the cytoplasm: one source containing a fixed and irreducible amount of the enzyme regardless of cell size whereas the amount of enzyme in the other source would vary with the amount of cytoplasm. Lactic and malic dehydrogenases are known to be localized exclusively in the cytoplasm of the liver and heart-muscle cells of rats, and, since amongst mammals the intracellular localization of a particular enzyme is usually the same in all tissues and even in all species, it would seem that the second interpretation of the regression lines for lactic and malic dehydrogenases of human tracheobronchial epithelia is the more probable. Some support for the rather tenuous suggestion that the epithelial cells have two sources of each dehydrogenase in their cytoplasm is provided by the recent demonstration of two individual cytoplasmic malic dehydrogenases in rat liver (Delbruck, Schimassek, Bartsch & Bucher, 1959; Thorne, 1960).

The cytological composition of the specimens used in the calculation of the regression lines varied widely: the extremes were, on the one hand, two cases showing extensive basal-cell metaplasia and two cases showing extensive squamous metaplasia (columnar/basal cells, 0.5), and, on the other hand, several cases, including one with pneumonia, showing increased numbers of goblet and ciliated cells (columnar/basal cells, 1.5). It is concluded from the high correlation coefficients of the regression lines that acid phosphatase, β -glucuronidase, arylsulphatases A plus B, arylsulphatase C and lactic and malic dehydrogenases are not therefore specifically involved in the differentiation of basal epithelial cells into columnar or squamous cells and that these enzymes are concerned in the general metabolism rather than in any specialized function of the epithelial cells. This implies that none of the enzymes mentioned is directly concerned in cilia action or in mucus production, and this implication was borne out by the lack of correlation of any of the enzyme activities with the number of ciliated or goblet cells in the various

Fig. 7. Variation of the average cytoplasmic volume of cells of bronchial epithelia with their ribonucleic acid content.

specimens. The origin of bronchial carcinomas is the basal cells of the bronchial epithelium, and the finding that the enzyme activities of the five surgically removed carcinomas and the two transplants in hamster cheek-pouch fit reasonably well on the regression lines strongly suggests that the enzymes examined are not specifically involved in the differentiation of the normal basal cells to neoplastic cells. It is further significant that, in the calculation of the regression lines, the enzyme activities of the epithelia from both the trachea and bronchus were included. The values for these samples (Figs. 1-6) suggest that the effect of tobacco smoke on the enzymes in the epithelia is the same in both trachea and bronchus and that this represents a non-specific rather than a carcinogenic effect of tobacco. This finding parallels the cytological studies (Ide *et al.* 1959), which have shown that smoking brings about the same cytological changes in both bronchial and tracheal epithelia.

The basis of the choice of enzymes for testing this method of study was mainly that of expediency (Spencer, 1959), and it was unfortunate that none of the enzymes proved to be of direct interest as far as bronchial carcinoma was concerned. Some of these enzymes have been said by others, on the basis of various experimental results, either to rise or fall in carcinoma when compared with the tissue of origin. These conclusions have been drawn with various parameters for the expression of enzyme activities such as wet weight of tissue or nitrogen content. However, as shown in this study, no difference was seen between normal and tumour tissue when the enzyme activities were placed on a cytoplasmic basis. It is suggested that in biochemical studies on tumours more emphasis should be placed on cytological control of the samples examined.

SUMMARY

1. Epithelia from the bronchus and trachea of human males over the age of 40 were removed within 6 hr. *post mortem* and stored at -20° as suspensions in 0.25 M-sucrose containing 0.01 M-ethylenediaminetetra-acetate, pH 7.4. Clinical histories and smoking habits of the subject were noted.

2. The acid-phosphatase, β -glucuronidase, arylsulphatases A plus B, arylsulphatase C and lactic and malic-dehydrogenase activities of these suspensions were assayed and correlated with total and differential cell counts, protein nitrogen and deoxy-ribonucleic acid and ribonucleic acid contents.

3. The activities of all the enzymes showed good correlation with the ribonucleic acid content of the epithelial cells, the regression lines having correlation coefficients of between 0.54 and 0.94. There

was no significant correlation between enzyme activities and any of the other parameters.

4. The enzyme activities of suspensions of surgically removed bronchial carcinomas (five specimens) and human bronchial carcinoma transplants in hamster cheek-pouch (two specimens) fitted reasonably well on the regression lines.

5. Analysis of these results suggested that all the enzymes examined are of cytoplasmic occurrence, that they are not directly involved in cilia action or mucus production and that they are not specifically involved in the differentiation of the basal epithelia cells into columnar, squamous or neoplastic cells.

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