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THE EFFECTS OF LONG-TERM EXPOSURE OF LUNG FIBROBLAST STRAINS TO CHRYSOTILE ASBESTOS

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Summary.—Evidence is presented that persistent long-term exposure of a strain of lung fibroblast-like cells to chrysotile asbestos over 37 passages *in vitro* leads to enhancement in cell mat collagen deposition and is accompanied by other metabolic alterations in the cultures.

ALTHOUGH IT has been known for many years that exposure to asbestos minerals can lead to lung fibrosis, which in some cases is progressive, the mechanism by which this disease develops is unclear.

In an attempt to understand the biochemical changes associated with asbestos-induced fibrogenesis *in vitro*, previous studies have concentrated on the short-term effects of asbestos on cultured lung fibroblasts. It has been shown that exposure to chrysotile asbestos induces both morphological (Richards and Jacoby, 1976; Richards *et al.*, 1974) and biochemical alterations (Richards and Morris, 1973) in lung fibroblast cultures. In particular, studies by Hext and Richards (1976) have shown that all the important commercial groups of asbestos can directly interfere with the process of fibrogenesis *in vitro* by raising or lowering the cell mat collagen levels of exposed fibroblast cultures, the observed effect being dependent upon the initial concentration of exposure and the type of asbestos employed.

These investigations, however, only constitute a short-term study of cellular exposure to asbestos (3 weeks) whereas *in vivo*, as a result of dust inhalation, lung cells may be continuously exposed for a number of years. In an attempt to duplicate the *in vivo* situation more closely, an investigation was undertaken to determine the effects of constantly exposing a line of lung fibroblast-like cells to a small dose of UICC Rhodesian chrysotile asbestos for a period of 37 passages (subcultures). Particular attention was given to the levels of cell mat fibrous collagen, RNA, DNA and protein in the asbestos-treated line and comparison was made with an identical normal line of cells which was undergoing a natural ageing process through 37 passages.

MATERIALS AND METHODS

Cell cultures.—A primary culture of rabbit lung fibroblast-like cells was isolated and grown as described previously (Richards, Wusteman and Dodgson, 1971). At the first subculture (Passage 1), the line was divided into two strains; one strain (D70/C) was given a small dose of UICC Rhodesian chrysotile asbestos in the majority of passages (see Fig. 3), whilst the other strain received no treatment and was retained as a control. Each strain was subcultured at regular intervals (every 6 or 7 days) and asbestos added to the chrysotile strain (D70/C) 2 or 3 days after subculture. At intervals, test cultures derived from both strains were set up for biochemical analysis by seeding approximately $0{\cdot}4 \times 10^6$ cells in medical flat bottles (100×40 mm). These cultures were maintained for 24 days (the test cultures derived from the chrysotile strain, D70/C, receiving no further asbestos treatment) and analysed as detailed below. The culture medium, 10 ml of 20% foetal bovine serum plus Waymouth's medium containing additional ascorbic acid (final concentration 50 μg/ml) and antibiotics (Richards *et al.*, 1971) was replaced twice weekly.

Asbestos dust.—The sample of Rhodesian chrysotile used was a standard reference sample prepared under the auspices of the International Union against Cancer (UICC). The dust was heat-sterilized (140° for 2 h), suspended in medium 199 by shaking vigorously for 10 s and then added to appropriate cultures as a single dose (1 ml).

Histological techniques.—Cultures were fixed in glutaraldehyde (1·25%) and stained (Giemsa) as described by Richards and Jacoby (1976).

Assays.—Prior to analysis cell cultures were treated as described previously (Richards *et al.*, 1971). DNA was estimated by the method of Burton (1956) as modified by Richards (1974) and RNA by the method of Ceriotti (1955). Cell protein was determined by the method of Oyama and Eagle (1956) and cell mat hydroxyproline (a determination of fibrous collagen) by the method of Stegeman (1958) after hydrolysis in 6M HCl for 24 h at 110°. All of the above assays were performed on individual test cultures and the results given in Figs. 3 and 4 represent the mean value obtained from at least duplicate cultures.

Media and chemicals.—Foetal bovine serum and medium 199 were purchased from Flow Laboratories Ltd., Irvine, Scotland, U.K. and Waymouth's MB 752/1 powdered medium from Burroughs Wellcome, Beckenham, Kent, U.K. (This latter product is no longer supplied by this company.) All chemicals were of " AnalaR " grade and were purchased from B.D.H. Ltd., Poole, Dorset, U.K.

RESULTS AND DISCUSSION

The cells of the normal strain (D70/N) retained typical fibroblast morphology through 37 passages (Fig. 1a–f) although growth was poor after this passage number and the strain could no longer be maintained after the 44th passage. In addition, after the 37th passage the cell sheets in this cell strain tend to " peel off " the glass surface of the culture bottle after 14 days. A similar problem of " peeling off " is found with the chrysotile-treated strain after the 40th passage. However, this strain of cells showed no deterioration in either growth ability or plating efficiency but was eventually abandoned after the 45th passage as there were no longer any control strains for comparative biochemical studies. Changes in morphology, as observed by light microscopy following short-term exposure of fibroblasts to chrysotile asbestos, have been reported previously (Richards and Jacoby, 1976) and similar changes are seen in the present investigation (Fig. 2a–f). Binucleate cells are common in the early passage numbers in the chrysotile-treated strain (Fig. 2d). In addition, between Passage 6 and Passage 25 in this strain there is an increase in the number of cells containing large nuclei, this effect being particularly noticeable at Passage 16 (Fig. 2e). Cells containing large nuclei are extremely rare in the early passage numbers of the normal strain but are more common after Passage 27 (Fig. 1e–f).

The levels of hydroxyproline, protein, DNA and RNA in 24-day-old cultures of the normal (D70/N) and chrysotile-treated (D70/C) fibroblast strains are shown (Fig. 3 and 4). The passage numbers at which dust was omitted from Strain D70/C are shown at the base of Fig. 3. Chrysotile produces toxic effects in fibroblast cultures (Richards and Jacoby, 1976) and therefore the omission of the asbestos at these times was considered necessary to enable the cell strain to be maintained in an actively growing state. In addition, the dose of asbestos given to the strain was lowered after Passage 14 to reduce the toxic effects to a minimum. The cell mat hydroxyproline levels (a measurement of fibrous collagen) of the normal strain show an initial peak followed by a gradual reduction to relatively low levels (Fig. 3). The level then generally increases between Passages 13 and 29, after which it gradually declines for a second time. It is perhaps important to point out that these normal cells are set up under identical conditions and cultured for the same time period, and yet the amount of fibrous collagen (measured as hydroxyproline) detected varies widely between different passages, although duplicate cultures from any one passage are very similar. The amount of cell mat hydroxyproline recorded at Passage 10 (6 μg/culture) is over 13 times lower than that found at Passage 29 (80 μg).

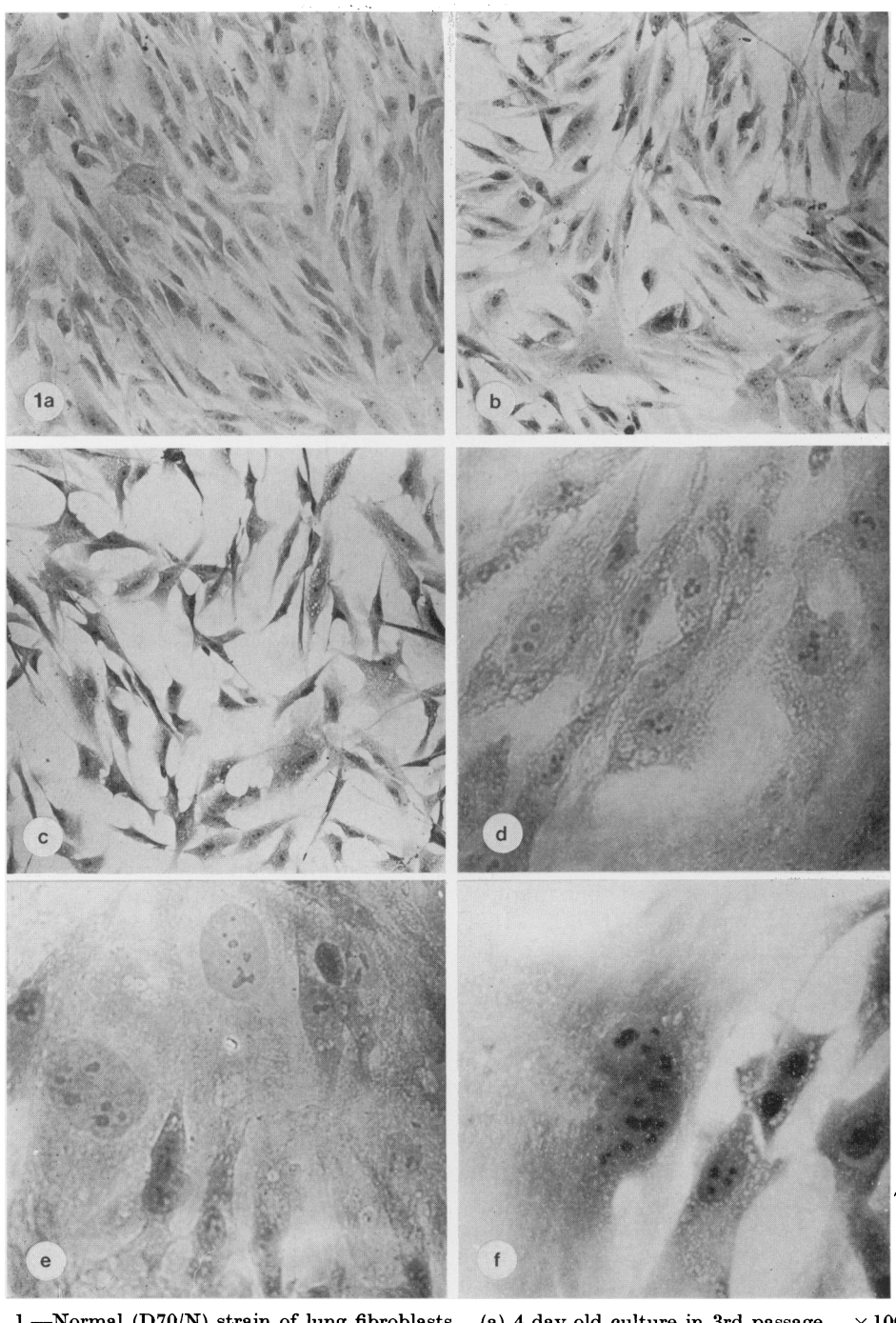

FIG. 1.—Normal (D70/N) strain of lung fibroblasts. (a) 4-day-old culture in 3rd passage. ×100. (b) 1-day-old culture in 26th passage. ×100. (c) 2-day-old culture in 36th passage. ×100. (d) 2-day-old culture in 21st passage. Phase contrast. ×400. (e–f) 1-day-old cultures in 27th and 31st passage respectively showing large nuclei. Phase contrast. ×400. Glutaraldehyde (1·25%) fix. Giemsa.

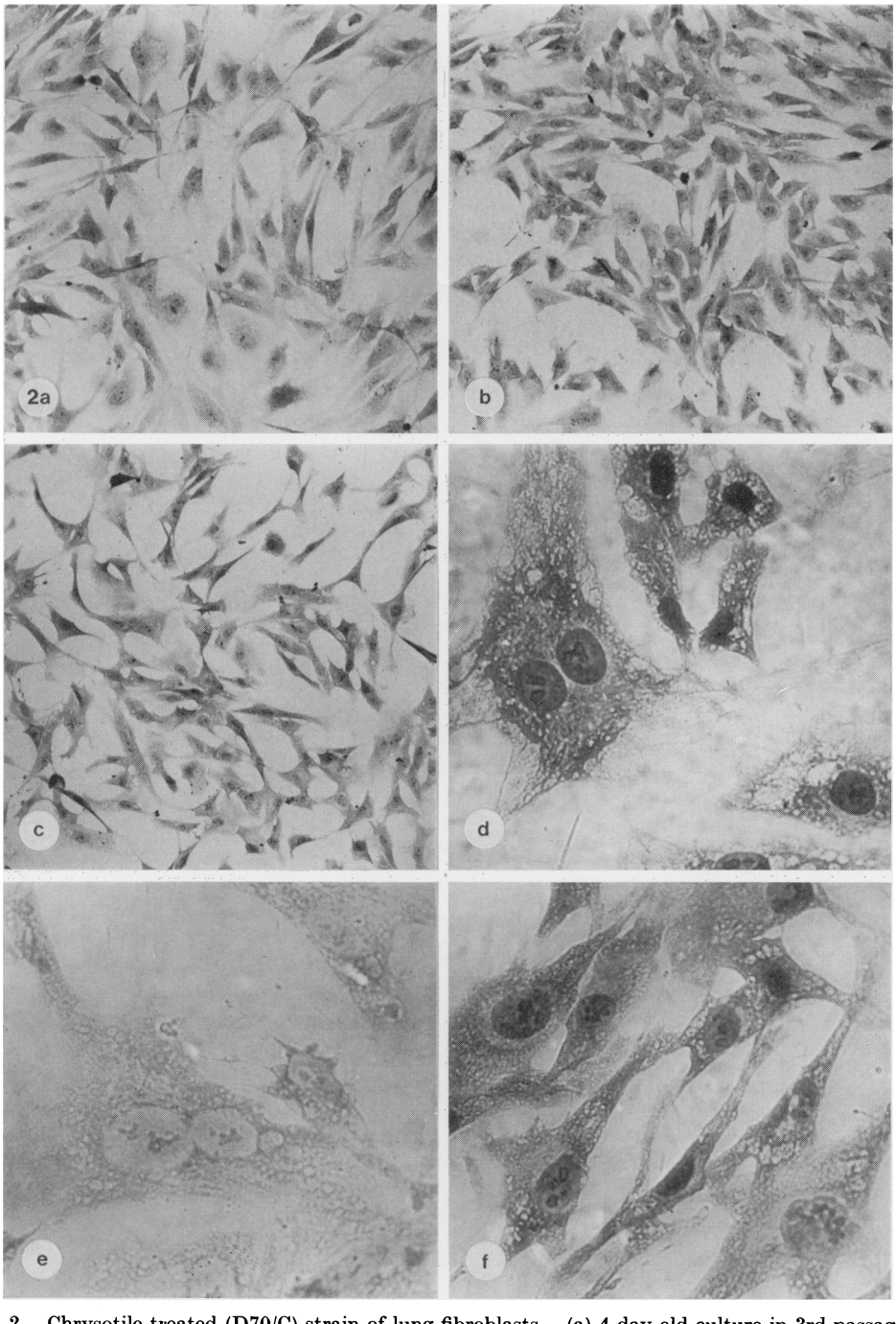

FIG. 2.—Chrysotile-treated (D70/C) strain of lung fibroblasts. (a) 4-day-old culture in 3rd passage. ×100. (b) 1-day-old culture in 23rd passage. ×100. (c) 1-day-old culture in 31st passage. ×100. (d) 3-day-old culture in 6th passage showing binucleate cell. Phase contrast. ×400. (e) 2-day-old culture in 16th passage showing large nuclei. Phase contrast. ×400. (f) 2-day-old culture in 35th passage. Phase contrast. ×400. Glutaraldehyde (1·25%) fix. Giemsa.

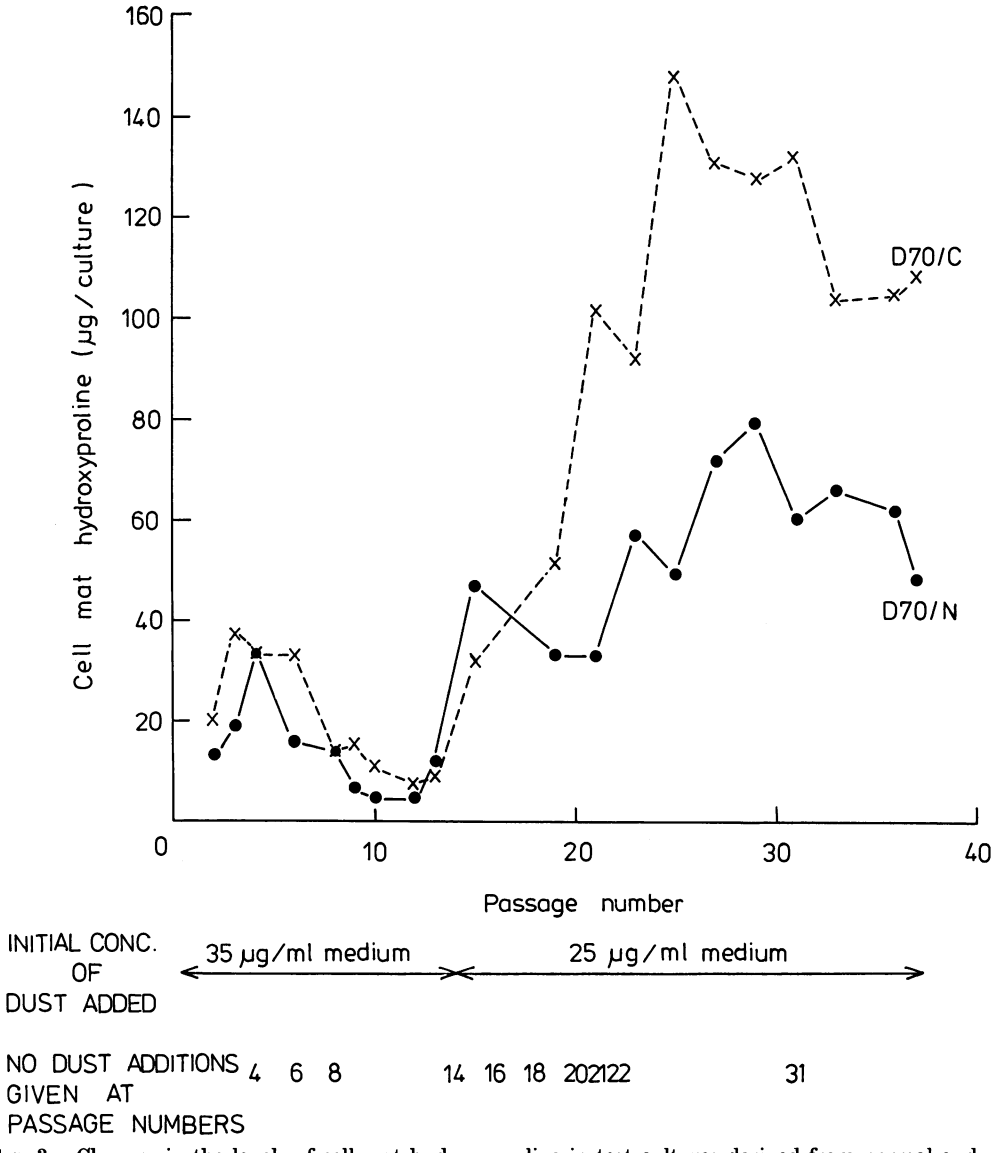

FIG. 3.—Changes in the levels of cell mat hydroxyproline in test cultures derived from normal and chrysotile-treated strains of lung fibroblasts. (For details see text. × --- ×, chrysotile-treated strain D70/C; ● ——— ● normal strain D70/N.)

The chrysotile-treated strain (D70/C) shows a similar pattern of change in hydroxyproline levels as the normal. However, the former cultures have higher levels of fibrous collagen per culture at each subculture with the exception of Passages 4, 13 and 15 (Fig. 3). Once again the variation of levels between different passage numbers may be enormous, *e.g.* the amount of hydroxyproline detected at Passage 12 (8 µg/culture) is over 18 times lower than found at Passage 25 (148 µg/culture). The differential between normal and chrysotile-treated strains is still maintained if hydroxyproline levels are expressed in terms of µg DNA or mg protein, thus indicating that the excessive accumulation of fibrous collagen by

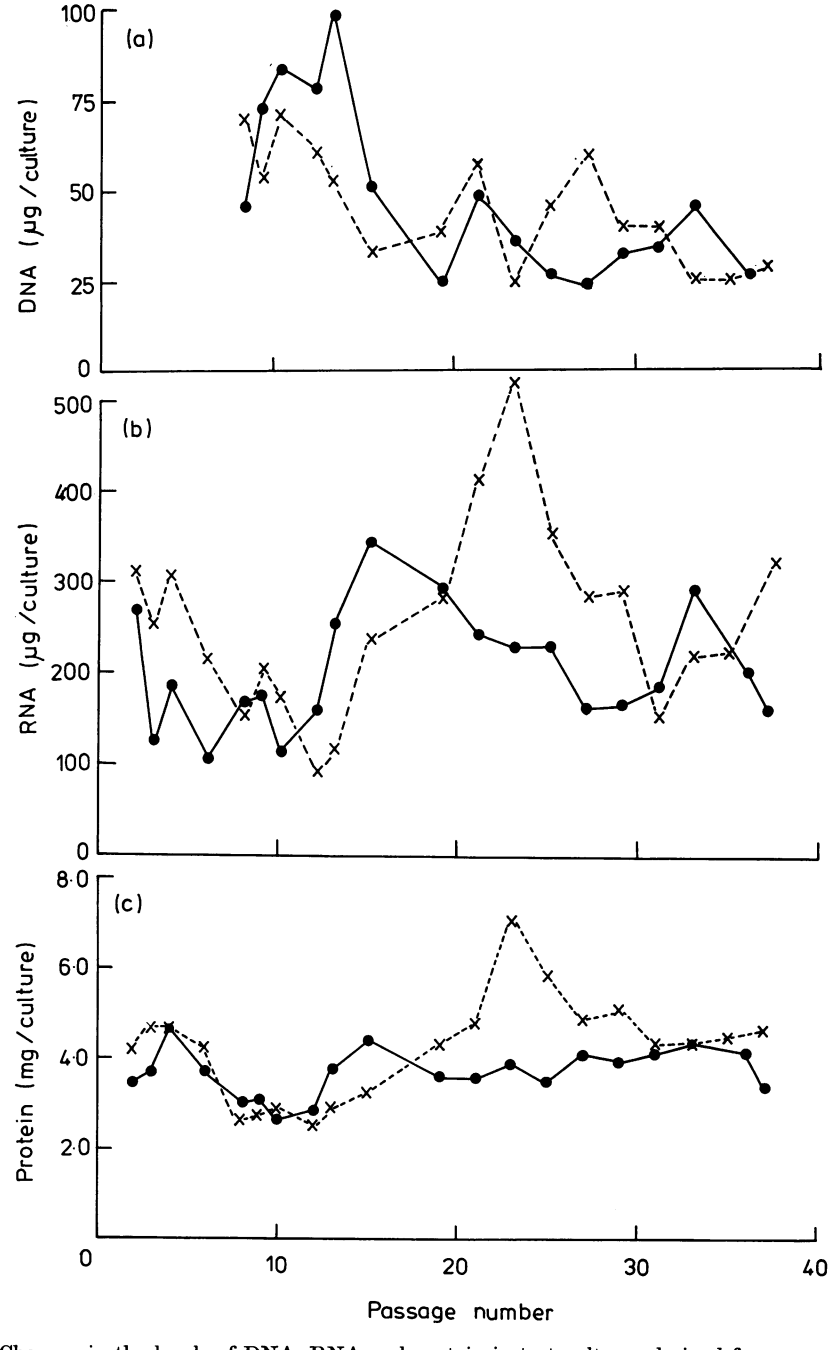

FIG. 4.—Changes in the levels of DNA, RNA and protein in test cultures derived from normal and chrysotile-treated strains of lung fibroblasts. (For details see text. × --- ×, chrysotile-treated, ● ——— ● normal.)

asbestos-treated cultures is not simply explained by an increase in cell numbers. In fact, DNA levels are often lower in the asbestos-treated strain than those found in the normal cultures (Fig. 4a) with notable exceptions at Passage Nos. 25–27. These results suggest that chrysotile asbestos exerts a relatively specific effect on the synthesis and/or deposition of collagen by fibroblast cells.

The actual amount of chrysotile asbestos to which the cultures taken for analysis are exposed is quite small, although possibly accumulative. The results in Figs. 3 and 4 are obtained from cultures which have received no further exposure to asbestos after their subculture from the chrysotile-treated strain (for details see Materials and Methods) and in many instances (Passages 4, 6, 8, 14, 16, 18 and 20–22) this strain itself received no dust treatment. Furthermore, a sub-strain (D70/C1) was derived from the asbestos-treated strain (D70/C) at the 38th passage and was maintained to the 45th passage without further exposure to asbestos. The levels of cell mat hydroxyproline in subcultures derived from this strain (D70/C1) were practically identical to those found in the asbestos-treated strain (D70/C) and thus were considerably higher than those found in equivalent normal (D70/N) cultures after 14 days *in vitro* (data not given). Thus it seems apparent that very small quantities of asbestos retained by some of a fibroblast population through many generations can directly stimulate synthesis/deposition of collagen and that further exposure to the mineral may be unnecessary in maintaining this fibrogenic response.

Changes in the levels of protein and RNA in normal and asbestos-treated strains are shown in Figs. 4b, 4c. In both strains RNA levels are usually related to protein levels and this would be expected as protein synthesis is an RNA dependent process. Both protein and RNA levels are considerably higher in the chrysotile-treated strain between Passages 20 and 27 than in the normal strain (Fig. 4b–c) and correspond to the considerably elevated levels of hydroxyproline found at this time in the former cultures (Fig. 3). However, after Passage 27 RNA and protein levels in the chrysotile-treated strain are similar to those of the normal strain, although fibrous collagen is still maintained at a high level in the former cultures. This again indicates a specific effect of chrysotile asbestos on collagen production/deposition by fibroblast cells, but the manner in which this effect is mediated is not known. Short-term studies exposing fibroblast cultures to different forms of asbestos (Hext and Richards, 1976) have indicated that these mineral dusts interfere with the secretion and/or synthesis of proteoglycans secreted into the culture medium and several workers (Obrink, 1973; Oegama *et al.*, 1975; Motomiya *et al.*, 1975) have stressed the importance of proteoglycan balance in the formation and deposition of fibrous collagen. The production of collagen and proteoglycan synthesis (Kurtz and Stidworthy, 1975) are both RNA-dependent processes. This has led to the suggestion that chrysotile asbestos produces alterations in RNA metabolism in fibroblast cells which precedes imbalance in proteoglycan production and release followed by an alteration in synthesis/deposition of collagen (Hext and Richards, 1976).

In summary, the results of the current *in vitro* investigation indicate that if lung fibroblast-like cells are persistently exposed to small quantities of chrysotile asbestos their capacity to synthesise and/or deposit fibrous collagen is considerably enhanced, an effect which may not be reduced for many cell generations following the cessation of exposure to the mineral. The relevance of such findings to the biochemical events which occur during the development of asbestos-induced fibrosis *in vivo* can only be surmised. In the lung the contact between asbestos and interstitial fibroblasts may be achieved by mineral

penetration of the alveolar walls, as suggested for silica by several investigators including Webster (1960). Alternatively, the release of a factor from dust-affected alveolar macrophages similar to that proposed for silica-induced fibrogenesis (Heppleston, 1971) may attract fibroblast cells to areas of dust deposition. The persistent reaction of small numbers of asbestos fibres either free or released from dying macrophages with fibroblast cells may be sufficient stimulus for a progressive fibrotic reaction.

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REFERENCES

BURTON, K. (1956) A Study of the Conditions and Mechanisms of the Diphenylamine Reaction for the Colorimetric Estimation of Deoxyribonucleic Acid. *Biochem. J.*, **62**, 315.

CERIOTTI, G. (1955) Determination of Nucleic Acids in Animal Tissues. *J. biol. Chem.*, **214**, 59.

HEPPLESTON, A. G. (1971) Observations on the Mechanism of Silicotic Fibrogenesis. In *Inhaled Particles, III Vol. 1. Proc. Int. Symp., London 1970.* Ed. W. H. Walton, Surrey: Unwin Brothers Ltd.

HEXT, P. M. & RICHARDS, R. J. (1976) Biochemical Effects of Asbestiform Minerals on Lung Fibroblast Cultures. *Br. J. exp. Path.*, **57**, 281.

KURTZ, M. J. & STIDWORTHY, G. H. (1975) Control over Acid Mucopolysaccharide Production of Cells *In Vitro*. *Biochim. biophys. Acta*, **399**, 90.

MOTOMIYA, M., HIDEO, A., SATO, H., YOKOSAWA, A., NAGAI, H. & KONNO, K. (1975) Increase of Dermatan Sulphate in a Case of Pulmonary Fibrosis. *Tohoku J. exp. Med.*, **115**, 361.

OBRINK, B. (1973) The Influence of Glycosaminoglycans on the Formation of Fibres from Monomeric Tropocollagen *In Vitro*. *Eur. J. Biochem.*, **34**, 129.

OEGAMA, T. R., LAIDLAW, J., HASCALL, V. C. & DZIEWIATKOWSKI, D. D. (1975) The Effect of Proteoglycans on the Formation of Fibrils from Collagen Solutions. *Arch. biochim. Biophys.*, **170**, 698.

OYAMA, V. I. & EAGLE, M. (1956) Measurement of Cell Growth in Tissue Culture with a Phenol Reagent (Folin-Ciocalteau). *Proc. Soc. exp. Biol., (N.Y.)*, **91**, 305.

RICHARDS, G. M. (1974) Modification of the Diphenylamine Reaction giving increased Sensitivity and Simplicity in the Estimation of DNA. *Analyt. Biochem.*, **57**, 369.

RICHARDS, R. J., HEXT, P. M., BLUNDELL, G., HENDERSON, W. J. & VOLCANI, B. E. (1974) Ultrastructural Changes in Lung Fibroblast Cultures exposed to Chrysotile Asbestos. *Br. J. exp. Path.*, **55**, 275.

RICHARDS, R. J. & JACOBY, F. (1976) Light Microscope Studies on the Effects of Chrysotile Asbestos and Fibre Glass on the Morphology and Reticulin Formation of Cultured Lung Fibroblasts. *Environ. Res.*, **11**, 112.

RICHARDS, R. J. & MORRIS, T. G. (1973) Collagen and Mucopolysaccharide Production in Growing Lung Fibroblasts exposed to Chrysotile Asbestos. *Life Sciences*, **12**, 441.

RICHARDS, R. J., WUSTEMAN, F. S. & DODGSON, K. S. (1971) The Direct Effect of Dusts on Lung Fibroblasts Grown *In Vitro*. *Life Sciences*, *10*, 1149.

STEGEMAN, H. (1958) Microdetermination of Hydroxyproline with Chloramine-T and p-dimethylaminobenzaldehyde. *Z. physiol. Chem. (Hoppe Seyler's)*, **311**, 41.

WEBSTER, I. (1960) Some Aspects of the Pathology of Silicosis Found in Miners from the South African Gold Mines. In *Proceedings of the Pneumoconiosis Conference, Johannesburg 1959.* Ed. A. J. Orenstein. London: J. & A. Churchill Ltd.