Virchows Arch A (1987) 411:403–408

Virchows Archiv A

Asbestos induced fibrosis in the omentum of rats

Immunofluorescence microscopical demonstration of collagens types I and III; laminin and fibronectin

J. Friemann[1], B. Voss[2], W. Weller[1], and K.-M. Müller[1]

[1] Universitätsklinik Bochum, Institut für Pathologie der Berufsgenossenschaftlichen Krankenanstalten „Bergmannsheil", D-4630 Bochum, Federal Republic of Germany

[2] Institut für Arteriosklerosеforschung an der Universität, D-4400 Münster, Federal Republic of Germany

Summary. Fibrosis and neoplasia are evoked by asbestos fibres. Different experimental models have been used to elucidate the cellular mechanism of their pathogenesis but there is no report available dealing with the role of structural glycoproteins and collagens in the development of the fibrosis. The omentums of 20 female SPF-Sprague-Dawley rats were investigated 1, 2 and 6 months after intraperitoneal injection of 15 mg UICC reference samples of crocidolite, by light- and immunofluorescence microscopy. Using monospecific antibodies, laminin, collagens types I and III and fibronectin were localized. After 6 months typical asbestos bodies were detected. By indirect marking of the basal lamina with anti-laminin-antibodies the marked degree of vessel proliferation occurring during the development of granuloma became visible. The deposition of connective tissue which was already established after 4 weeks was mainly due to collagen type III. After 4 and 8 weeks an accumulation of fibronectin associated with larger asbestos fibres was observed. The results suggest that fibrogenesis is promoted by the opsonic activity of fibronectin for long asbestos fibres. The fibrosis may derive from activated resident fibroblasts.

Key words: Crocidolite asbestos – Rat model – Laminin – Collagen – Fibronectin

Introduction

The development of lung fibrosis after asbestos inhalation was first described by Cooke (1924). Wagner (1960) observed the coincidence of the formation of pleural and peritoneal mesotheliomas and asbestos inhalation. To discover the pathomechanism of asbestosis, asbestos dust was applied in different animal experiments either tracheally or intrapleurally, or intraperitoneally (Wagner 1980; Fasske 1986). The fibrogenic activity and cancerogenic activity of the fibres did not depend on the fibre types only, but also on the application method used and was closely related to fibre length, diameter and concentration of instilled asbestos fibres (Beck 1975; Davis 1979; Pott 1983).

Under experimental conditions asbestos fibres always induced the development of granulomas as a result of a foreign body reaction in the interstitial connective tissue which was infiltrated by monocytes, macrophages, multinucleated giant cells and lymphocytes (Davis 1970; Weller et al. 1984; Fasske 1986).

Apart from the inflammatory reaction towards asbestos, there may be involvement of fibronectin as a non-specific opsonin and chemotactic structural glycoprotein in the interstitial phase of fibrogenesis (Van de Water, III et al. 1981; Hynes et al. 1982; Hakansson 1985). Moreover, fibronectin influences the proliferation of fibroblasts in the young granulation tissue and mediates cell-cell and cell-matrix interactions (Tsukamoto et al. 1981; Yamada 1983). The deposition of collagens types I and III represents the degree of fibrous activity (Rauterberg 1981) while the basement membrane glycoprotein laminin indicates the extend of the vascularisation of the granulation tissue (Timpl et al. 1979). In the present contribution the development of granulomas after intraperitoneal injection of crocidolite asbestos was followed up by light microscopy after routine staining and immunohistochemistry using antibodies to collagens types I and III, laminin and fibronectin.

Offprint requests to: J. Friemann

Material and methods

4 months old female SPF-Sprague-Dawley rats of the Zentralinstitut für Versuchstierzucht in Hannover, FRG, were kept under hygienic conditions and were fed with standard diet (Sniff-R, 20 g per animal per day) and tap water ad libitum. Either 1 ml physiological NaCl solution as a control or 15 mg UICC reference samples of crocidolite (fibre lengths between 1.3 and 13.8 µm, kindly provided by Prof. F. Pott, Düsseldorf, FRG) (Fig. 1a) suspended in 1 ml physiological NaCl solution were injected into 20 animals, intraperitoneally.

1, 2 and 6 months after the dust injection two animals of each group were sacrified under narcotization. The omentum was divided into two parts. One part was fixed in formaldehyde 10% v/v solution, embedded in paraffin and sectioned for different routine staining.

The staining was performed with haematoxylin-eosin, Prussian blue, chromotrop-aniline blue (200 ml 0.2 NaCl, 4 g Chromotrop 2R, 1 g Anilinblau; pH 0.9–1.3) and Goldner (Goldner 1938).

The second part of the omentum was deep frozen and stored at −80° C for cryostat sections. For indirect immunofluorescence the deep frozen tissue was cut at −25° C into 6 µm sections which were attached to glass trays. To smoothen the sections the trays were dipped into phosphate buffered saline (PBS), pH 7.7, for 10 s. The sections were fixed by drying at 37° C. The tissue sections were incubated either with specific antibodies against fibronectin, collagens types I and III as well as laminin or with non-immune-serum for control for 30 min at room temperature (Voss and Rauterberg 1986). After the incubation the sections were rinsed five min in PBS. For the visualization of the first antibody a further incubation with fluorescein-isothiocyanate conjugated IgG (Behringwerke, Marburg, FRG) directed against the first antibody, was performed for 30 min at room temperature. To prevent an unspecific adsorbtion of antibodies the tissue sections were rinsed again in PBS according to the method described (Voss and Rauterberg 1986).

In order to quantify the changes or the absence of changes in the different time periods, granulomatous lesions of comparable size, cellularity and asbestos content were documented photographically. Afterwards the increase or decrease of cellularity and immunofluorescence intensity were estimated by comparison of different stages by two independent investigators.

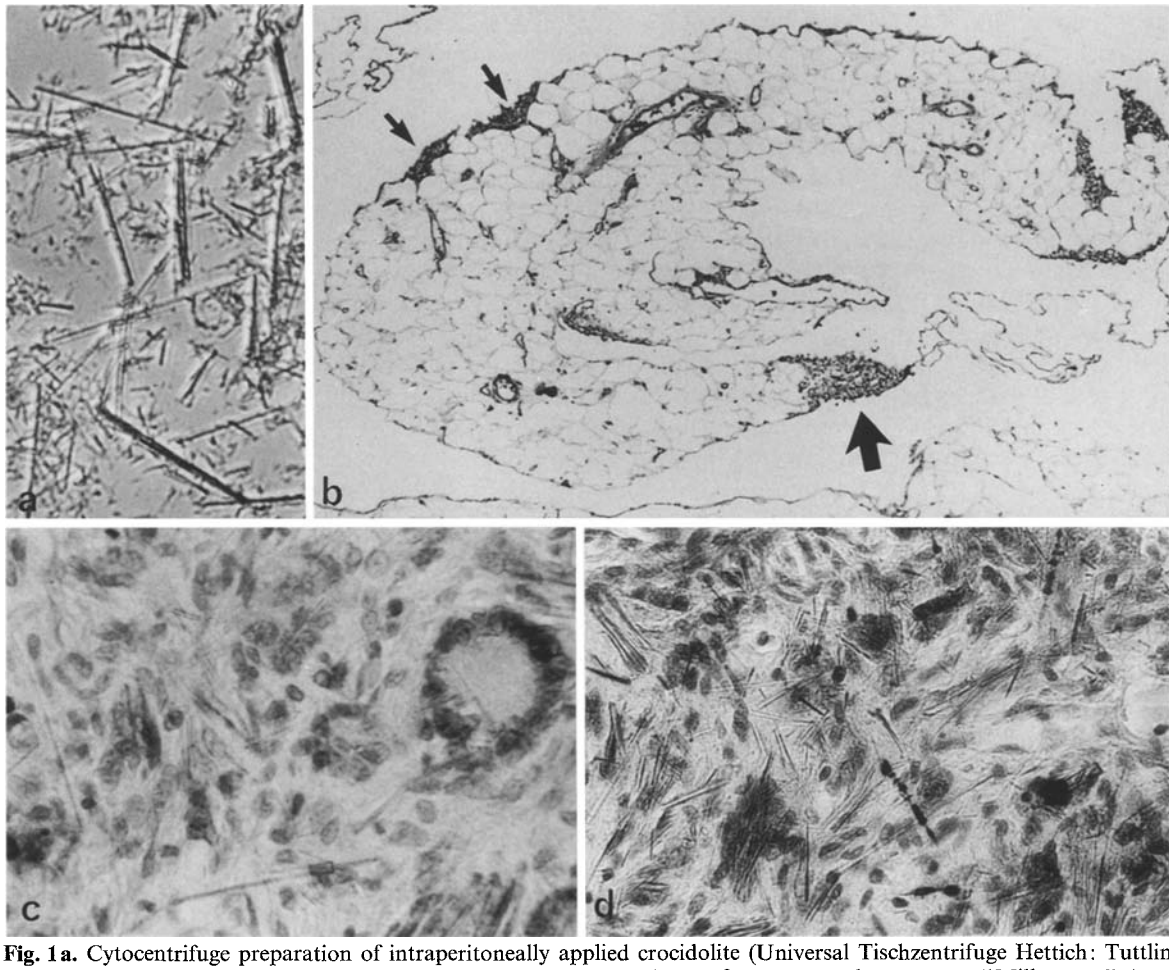

Fig. 1a. Cytocentrifuge preparation of intraperitoneally applied crocidolite (Universal Tischzentrifuge Hettich: Tuttlingen, FRG, 2000 rpm × 7 min) Light microscopy, polarisation ×250. **b** Fat tissue of rat's normal omentum. "Milk spots" (arrows) in the area of vessel branches. HE, light microscopy ×80. **c** Numerous mononuclear macrophages as well as giant cells of foreign body- and Langhans type partly ingesting asbestos fibres by phagocytosis in granulomas 4 weeks after application of 15 mg crocidolite intraperitoneally. HE, ×600. **d** Typical asbestos bodies of different stages in granulomas after 6 months. Light microscopy, Perls' iron stain. ×320

Results

The normal omentum of the rat consists of a loose network of connective tissue fibres. Typical lymph nodes with a fibrous capsule were not observed, although in the area of the blood vessel branches nodular accumulations of cells belonging to the mononuclear cell system were present. These "milk spots" could be observed by haematoxylin-eosin staining (Fig. 1 b).

4 weeks after the fibre application the haematoxylin-eosin staining revealed sharply demarked granulomas of different sizes preferably in the area of "milk spots". The granulomas consisted of macrophages, multinuclear giant cells, fibroblasts and some round cells (Fig. 1 c). Bundles of crocidolite fibres, easily visible after HE staining, were distributed predominantly in the interstitial spaces but partially occurred intracellularly. A significant alteration of the cellular composition within the granulomas was not observed after 2, 4, and 6 months.

The deposition of the connective tissue was mainly concentrated in granulomatous lesions but was also visible in cordlike masses under the serosa. The fibrotic scar formations of the granulomas were already established 4 weeks after the dust injection. There was only a slight further fibre deposition observed between 1 and 6 months in CAB-staining although the cellular content of the granulomatous lesions was not diminished. Within this time neither general activation of the connective tissue in the adjacent omentum nor degeneration in the dense granulomas could be seen.

The specific Prussian blue reaction for ferrous material showed a weak staining along bundles of

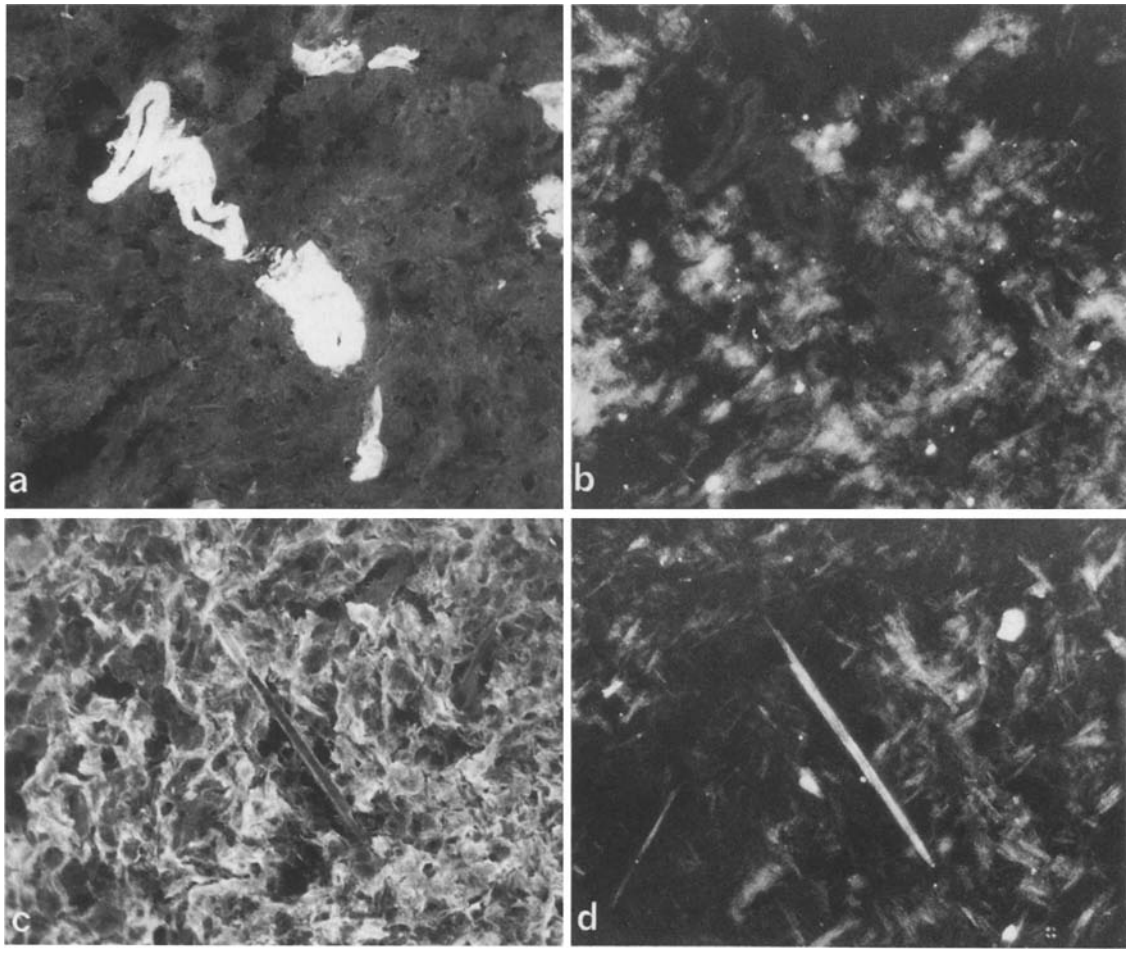

Fig. 2a. Immunofluorescence microscopy of laminin in basement membranes of capillaries in a granuloma. ×150. **b** Polarisation microscopy of the same area of (**a**). Crocidolite fibres are visualized in the white dots and spots. ×150. **c** Immunofluorescence microscopy of a granuloma stained specifically for collagen type III. The granuloma developed 4 weeks after dust application. ×600. **d** Polarisation microscopy of (**c**) with a bundle of long crocidolite fibres. ×600

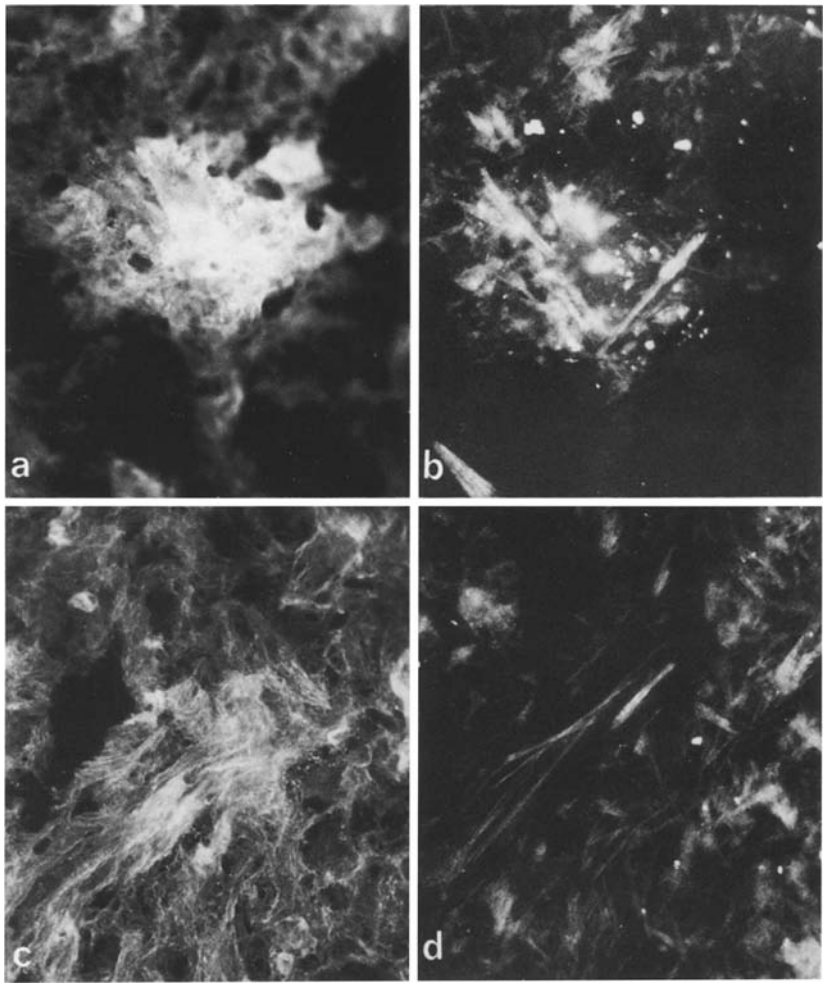

Fig. 3a–d. Indirect immunofluorescence of fibronectin (**a**) associated with clustered crocidolite fibres visualized by polarisation microscopy (**b**). Long crocidolite fibres are opsonized by fibronectin (**c**). They became visible by polarisation microscopy (**d**). × 600

crocidolite. The iron obviously derived from both the asbestos fibres themselves and ferrous positive contaminations. Typical asbestos bodies were detected in the stage after 6 months (Fig. 1d).

In the lobules of organized fat tissue of the omentum collagens types I and III are present in a reticular network. Larger depositions were observed in the adventitial layer of blood vessels. A weak positive fluorescence for fibronectin was visible all over the omentum. The vascularisation could be illustrated by the strong fluorescence specific for laminin.

The marked degree of vessel proliferation occuring during granuloma development became visible after indirect marking the basal lamina with anti-laminin antibodies (Fig. 2a and b). 4 and 8 weeks after i.p. injection of asbestos fibres it was more intensive than at the end of the 6-months experimental period. From light microscopy fibres labeled with antibodies against collagens types I and III formed dense scar formations in granulomas of different size and different stage (Fig. 2c and d). Already after 4 weeks both collagen types were co-distributed and during the observation period up to 6 months we saw only slight dynamic changes in the collagen types I and III ratio. Collagen type I stained less intensively and increased later than collagen type III. There was no preference for one special collagen type near the bundles of crocidolite fibres. An accumulation of fibronectin associated with larger asbestos fibres was observed (Fig. 3). It decreased clearly until 6 months. Yet, fibronectin depositions on asbestos fibres similar to the segmental coat of asbestos bodies could not be found.

Discussion

Macrophages regularly participate in the early stages of experimental asbestosis in animals. In an asbestos-mediated activation of the immune response, peripheral monocytes are attracted chemotactically and become activated macrophages which attack the asbestos fibres (Tetley et al. 1976;

Davis et al. 1985; Warheit et al. 1985). Short fibres (smaller than 3 μm) are rapidly incorporated by invagination of the macrophage membrane and transported to the local mesenteric lymph nodes (Beck et al. 1971; Davis 1972; Weller et al. 1984; Fasske 1986). However, there is no agreement on the question what happens to longer asbestos fibres (>10 μm). Beck (1975; 1983) discussed an incomplete phagocytosis of the fibres with penetration of the macrophage membrane. Other authors described attachment of multinuclear giant cells and mononuclear macrophages to long fibres (Davis 1972; Hilscher 1972; Weller et al. 1984). Fasske (1986) suggested that the larger fibres are broken down in the macrophages into small fragments of less than 0.01 μm.

There is no doubt that delayed phagocytosis of the longer asbestos fibres is responsible for the persistence of granulomas for months. Yet, investigations concerning the mechanism of promoting phagocytosis are not available. In connection with complement C5a activation, a possible non-specific opsonic reaction has been discussed (Wilson et al. 1977; Clark and Klebanoff 1978; Saint-Remy et al. 1980; Bohnsack et al. 1985; Warheit et al. 1985). However, accumulation of fibronectin associated with bundles of crocidolite fibres may confirm earlier observations of the opsonic effect of this glycoprotein (Molnar et al. 1979; Gudewicz et al. 1980; Van de Water, III et al. 1981; Friemann et al. 1985).

During chronic inflammation in experimental asbestosis the involvement of fibronectin in fibrotic reactions is of particular interest. Cellular fibronectin produced by activated macrophages or plasma-fibronectin has a chemotactic and stimulating effect on fibroblasts (Tsukamoto et al. 1981). The environmental conditions for proliferation of the fibroblasts are supported by blood vessel proliferation. The specific fluorescence of collagen fibres is mainly due to collagen type III and derives either from a larger number of fibroblasts or increased collagen synthesis. Connective tissue formation may be activated by monokines or even by asbestos fibres themselves (Leibovich and Ross 1976). The present results suggest that opsonic activity of fibronectin for long asbestos fibres promotes fibrogenesis by activation of resident fibroblasts (Richards et al. 1971; Pernis et al. 1966).

References

Beck EG, Bruch J, Friedrichs K-H, Hilscher W, Pott F (1971) Fibrous silicates in animals experiments and cell-culture-morphological cell and tissue reactions according to different physical chemical influences. In: Walton WH (ed) Inhaled particles III, vol 1. Unwin Brothers Ltd, Old woking/Surrey, pp 477–486

Beck EG (1975) Biologische Wirkung von faserförmigen Stäuben. Arbeitsmed Sozialmed Präventivmed 10:178–183

Beck EG (1983) Zytotoxizität lungengängiger Stäube. Kongr Ber Wiss Tag Norddtsch Ges Lungen- und Bronchialheilk 18:393–400

Bohnsack JF, O'Shea JJ, Takahashi T, Brown EJ (1985) Fibronectin – enhanced phagocytosis of an alternative pathway activator by human culture-derived macrophages is mediated by the $C_{4b/3b}$ complement receptor (CR 1). J Immunol 135:2680

Clark RA, Klebanoff SF (1978) Role of the classical and alternative complement pathways in chemotaxis and opsonization: studies of human serum deficient in C_4. J Immunol 120:1102–1108

Cooke WE (1924) Fibrosis of the lungs due to the inhalation of asbestos dust. Br Med J 1924:147

Davis JMG (1970) The long term fibrogenic effects of chrysotile and crocidolite asbestos dust injected into the pleural cavity of experimentals animals. Br J Exp Pathol 51:617–627

Davis JMG (1972) Fibrogenic effects of mineral dust injected into the pleural cavity of mice. Br J Exp Pathol 53:190–201

Davis JMG (1979) Current concepts in asbestos fiber pathogenity. In: Lemen R (ed) Dusts and disease III. Park Forest South, pp 45–48

Davis JMG, Bolton RE, Cowie H, Donaldson K, Gormley IP, Jones AD, Wright A (1985) Comparisons of the biological effects by mineral fibre samples using in vitro and in vivo assay systems. In: Beck EG, Bignon J (eds) In vitro effects of mineral dusts. Springer, Berlin Heidelberg New York, pp 405–411

Fasske E (1986) Pathogenesis of pulmonary fibrosis induced by crysotile asbestos. Longitudinal light and electron-microscopic studies on the rat model. Virch Arch A (Pathol Anat) 408:329–346

Friemann J, Voss B, Müller K-M (1985) Fibronectin in silikotischen Granulomen. Verh Dtsch Ges Pathol 69:663

Goldner J (1938) A modification of the Masson trichrom technique for routine laboratory purpose. Am J Pathol 14:237–243

Gudewicz PW, Molnar J, Lai MZ, Beezhold DM, Siefring GE, Credo RB, Lorand L (1980) Fibronectin-mediated uptake of gelatin-coated latex particles by peritoneal macrophages. J Cell Biol 87:427–433

Hakansson L, Venge P (1985) The combined action of hyaloronic acid and fibronectin stimulates neutrophil migration. J Immunol 135:2735

Hilscher W (1972) Beiträge zur experimentellen Asbestose der Wistarratte. Zentralbl Allg Pathol 116:413–416

Hynes RO, Yamada KM (1982) Fibronectins: Multifunctional modular glycoproteins. J Cell Biol 95:369–377

Leibovich SJ, Ross R (1976) A macrophage-dependent factor that stimulates the proliferation of fibroblasts in vitro. Am J Pathol 84:501–514

Molnar J, Gelder FB, Lai MZ, Siefring GE, Credo RB, Lorand L (1979) Purification of opsonically active human and rat cold insoluble globuline (plasma fibronectin). Biochem 18:3909–3916

Pernis B, Vigliani EC, Marchisio MA, Zanardi S (1966) Observations on the effect of asbestos on cells 'in vitro'. Med Lav 57:721–729

Pott F (1983) Untersuchungen zur Ätiologie asbestbedingter Tumoren. Atemw – Lungenkrkh 9:372–375

Richards RJ, Wustemann FS, Dodgson KS (1971) The direct effects of dusts on lung fibroblasts grown in vitro. Life Sci 10:1149–1159

Rauterberg J, Jander R, Voss B, Furthmayr H, Glanville R (1981) Characteristics and occurrence of short chain collagen. In: Workshop conference Hoechst. Raven Press, New York

Saint-Remy JMR, Cole P (1980) Interactions of chrysotile asbestos fibres with the complement system. Immunology 41:431

Tetley TD, Hext PM, Richards RJ, Mc Dermott M (1976) Chrysotile induced asbestosis. Changes in free cell population, pulmonary surfactant and whole lung tissue of rats. Br J Exp Pathol 57:505–514

Timpl R, Rhode H, Robey PG, Remard SJ, Foidart J-M, Martin GR (1979) Laminin – a glycoprotein from basement membranes. J Biol Chem 254:9933–9987

Tsukamoto Y, Heisel WE, Wahl SM (1981) Macrophage production of fibronectin, a chemoattractant for fibroblasts. J Immunol 127:673–678

Van de Water III L, Schroeder LS, Crenshaw EB, Hynes RO (1981) Phagocytosis of gelatine latex particles by a murine macrophage line is dependent on fibronectin and heparin. J Cell Biol 90:32–39

Voss B, Rauterberg J (1986) Localization of Collagen Types I, III, IV and V, fibronectin and Laminin in Human Arteries by Indirect Immunofluorescence Method. Pathol Res Pract 181:568–575

Wagner JC, Sleggs CA, Marchand P (1960) Diffuse pleural mesothelioma and asbestos exposure in the north western cape province. Br J Ind Med 17:260–271

Wagner JC (ed) (1980) Biological effects of mineral fibres. Vol 1, IARC (International Agency for Research on Cancer) scientific publications No 30, Lyon

Warheit DB, George G, Hill LH, Synderman R, Brody AR (1985) Inhaled asbestos activated a complement-dependent chemoattractant for macrophages. Lab Invest 52:505–514

Weller W, Kissler W, Friedrichs K-H, Morgenroth K (1984) Fibrogenität ultramikroskopischer Chrysotil-Asbest-Fasern. In: Bergbau-Berufsgenossenschaft (ed) VI. Internationale Pneumokoniose-Konferenz 1983, Bochum. Wirtschaftsverlag NW, Verlag für neue Wissenschaft Bremerhaven, pp 1021–1027

Wilson MR, Gaumer HR, Salvaggio JE (1977) Activation of the alternative complement pathway and generation of chemotactic factors by asbestos. J Allergy Clin Immunol 60:218

Yamada KM (1983) Cell surface interactions with extracellular materials. Ann Rev Biochem 52:761–799

Accepted June 9, 1987