THE BIOLOGICAL EFFECTS OF THE INTERNATIONAL STANDARD REFERENCE ASBESTOS SAMPLES (UICC) ON THE LUNGS OF RATS*

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ve research has been undertaken concerning the al effects of asbestos in the lungs of different species.[1-6] In most experiments the chemical and l properties of the asbestos samples used were not ined. The results obtained under such conditions hampered the interpretation and comparison of ork of the different investigators.

he request of the International Union Against Cancer), Standard Reference Asbestos Samples were com- by the Pneumoconiosis Research Unit in Johannes- nd, recently released for research purposes. Almost chemical and physical properties of these samples been determined or will be available soon.

he present investigation a comparative histopatho- l study was undertaken to assess the biological effects 5 International Reference Asbestos Samples on the of rats.

MATERIALS AND METHODS

os Samples

UICC Standard Reference Asbestos Samples were

ocidolite	lot No. XI, 2
tophyllite	lot No. VII, 1
mosite	lot No. VII, 2
rysotile A	lot No. XVI, 3
hrysotile B	lot No. V, 2

s in our laboratory concerning the mean fibre length reference asbestos samples showed that significant nces exist. Because of the high percentage of long difficulties were also experienced with the injection dusts intratracheally. To perform the intratracheal injections successfully the individual samples were milled in an agate-ball mill to reduce the mean fibre length. The chrysotile B variety was exceptionally tough and the mean fibre length was only slightly reduced after 40 hours of grinding. The size distribution of the fibres in the different examples is given in Table I and the chemical composition in Table II.

TABLE I. FIBRE SIZE DISTRIBUTION OF THE ASBESTOS SAMPLES AFTER MILLING

Fibre size	*Crocidolite*	*Amosite*	*Antophyllite*	*Chrysotile A*	*Chrysotile B*
<5 μm	90%	98%	99%	100%	26%
5 - 10 μm	8%	2%	1%	—	28%
>10 μm	2%	—	—	—	46%
Mean fibre length (μm)	3·43	2·64	2·59	2·52	17·63

Animals

Albino rats (*Rattus norvegicus*, Wistar Institute) of the same age, weight (140 ± 10 g) and sex (female) were used.

Four groups of 25 animals each were injected with suspensions of crocidolite, amosite, antophyllite and chrysotile A respectively. The chrysotile B suspension was administered to 16 animals only.

Asbestos suspensions were prepared in isotonic saline in a concentration of 50 mg dust/1 ml saline. All the suspensions were sterilized.

Each animal was intratracheally injected with 1 ml suspension followed by 1 ml of air, according to the method described previously.[8] The chrysotile B suspension was administered in a dose of 25 mg/0·5 ml into one of the lungs of an animal only. This procedure was adopted to prevent the high mortality rate due to the abundance of long fibres in chrysotile B asbestos.

The experiment lasted 240 days and 5 rats from each

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TABLE II. THE CHEMICAL COMPOSITION OF UICC ASBESTOS SAMPLES[7]

Constituents	*Amosite*	*Antophyllite*	*Crocidolite*	*Rhodesian chrysotile (A)*	*Canadian chrysotile (B)*
Major constituents (%)	0·34 N	0·72 N	0·47 N	0·40 N	0·27 N
Aluminium	28 N	4·4 N	27 N	1·7 N	2·6 N
Iron	15·1 A	1·98 A	15·1 A	0·88 A	1·14 A
Magnesium	11·0 N	24 N	3·6 N	31 N	32 N
Oxygen					
Sodium	—	—	4·4 N	—	0·08 N
Sodium as Na_2O	0·11 F	0·08 F	5·8 F	0·04 F	0·02 F
Silicon as SiO_2	50·3 G	58·2 G	49·1 G	39·1 G	38·6 G
Free SiO_2	3·0 A	0·23 A	2·06 A	0·01 A	0·13 A
Minor constituents (ppm)					
Cobalt	7 N	50 N	2 N	55 N	46 N
	11 A	24 A	9 A	54 A	45 A
Chromium	35 N	870 N	16 N	1 390 N	490 N
	32 A	571 A	22 A	1 395 A	316 A
Manganese	15 000 N	1 700 N	870 N	430 N	510 N
	13 900 A	988 A	864 A	366 A	443 A
Nickel	100 N	1 360 N	100 N	1 250 N	990 N
	34 A	424 A	13 A	1 445 A	795 A
Antimony	2 N	—	2 N	4 N	—
Scandium	5 N	5 N	0·5 N	6 N	5 N

= atomic absorption spectrophotometry; F = flame emission spectrophotometry; G = gravimetric analysis; N = neutron activation analysis

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group were killed at 60-day intervals (in the chrysotile B group only 4 rats were killed at each interval).

Histological Technique

Each animal was anaesthetized with ether and the trachea was exposed, and 7·5 ml of 10% formol-saline was injected into the lungs after the animal had been bled to death. The lungs were dissected free and fixed in 10% formol-saline.

After fixation, a 3-mm block of tissue was selected from each lung in a sagittal plane near the hilum, embedded in wax and sectioned serially (6 μm). Two sections were stained with haematoxylin and eosin and another was impregnated with silver.

The amount and grade of fibrosis were determined according to the system of Ross *et al.*[9]

RESULTS

Macroscopic Appearances

No marked macroscopic differences could be detected among the lungs from the different groups injected with short-fibre samples. The lungs were soft and adhesions between the parietal and visceral pleurae were quite common. This phenomenon was not observed in lungs from the chrysotile A group.

Lungs from the chrysotile B group could easily be distinguished from the other groups. The injected lung was usually hard and non-functional, while the non-injected lung had become considerably enlarged.

Microscopic Appearances

The mean grade and amount of fibrosis produced by the various asbestos samples in the lungs of rats at different time intervals over a period of 240 days are given in Table III.

All the lungs showed an initial macrophage mobilization at the 60-day stage. Most of the macrophages were concentrated in the alveoli adjoining the respiratory bronchioli and to a lesser extent in the peribronchiolar and perivascular lymphatics. In some respiratory bronchioli and alveoli an amorphous material was observed which usually contained asbestos fibres and occasionally macrophages.

A high incidence of infection was found in all the groups. The infection was usually localized in and around the greater bronchi and was accompanied by hyperplasia of the lymph nodules.

The grade and amount of fibrosis produced by the short-fibre asbestos samples were slight and consisted of isolated areas of diffuse reticular connective tissue (Fig. 1). Long-fibre asbestos (chrysotile B), in contrast, caused a more pronounced cellular reaction followed by a compact nodular fibrosis (Fig. 2).



Fig. 1. Diffuse reticular connective tissue, 60 days after intratracheal injection of short-fibre crocidolite (silver impregnation × 100).



Fig. 2. Tissue reaction 60 days after the intratracheal injection of long-fibre chrysotile (H & E × 100).

At the 120-day stage the number of macrophages decreased and phagocytosis of free asbestos fibres virtually completed. Areas of fibrosis were found, especially in the perivascular and peribronchiolar tissue, but the alveolar walls adjoining the respiratory bronchioli were also involved and quite often the visceral pleurae. The fibrosis elicited by short-fibre asbestos remained diffuse and consisted of reticular fibres with some collagen fibres in lung areas with chronic inflammation.

The chrysotile B fibres caused a nodular, compact fibrosis which became acellular at this stage. Abscesses were quite frequently observed in the lung parenchyma of this group, a phenomenon which might be due to occlusion of some of the large bronchioli.

TABLE III. MEAN GRADE, MEAN AMOUNT AND MEAN TOTAL FIBROSIS AFTER INTRATRACHEAL INJECTION OF VARIOUS ASBESTOS SAMPLES

Asbestos type	60 days			120 days			180 days			240 days			Mean 60 - 240 days		
	Grade	Amount	Total fibrosis	Grade	Amount	Total fibrosis	Grade	Amount	Total fibrosis	Grade	Amount	Total fibrosis	Grade	Amount	Total fibrosis
Amosite	1·20	1·30	1·69	1·13	1·40	1·60	1·60	1·40	1·40	1·10	1·20	1·80	1·10	1·33	1·[illegible]
Anthophyllite	1·10	1·50	1·70	1·03	1·30	1·30	1·10	1·10	1·20	1·30	1·10	1·40	1·13	1·25	1·[illegible]
Crocidolite	1·20	1·20	1·50	1·03	2·03	1·60	1·20	1·50	2·03	1·03	1·50	1·50	1·05	1·63	1·[illegible]
Chrysotile A	1·30	1·20	1·50	1·03	1·10	1·10	1·00	1·10	1·10	1·10	1·03	1·10	1·10	1·10	1·[illegible]
Chrysotile B	1·70	1·83	3·09	1·67	1·67	3·25	1·57	1·85	4·75	3·09	3·66	11·09	1·99	2·15	[illegible]

The histopathological picture of the lungs from the short-fibre asbestos groups did not show major progressive changes after 120 days. In fact, the number of macrophages and free unphagocytosed asbestos fibres were considerably reduced at 180 days. A progressive fibrosis persisted only in some lung areas where asbestos fibres were retained in the interstitial tissue. Lungs from the chrysotile A group appeared to be quite normal at the 180-day stage.

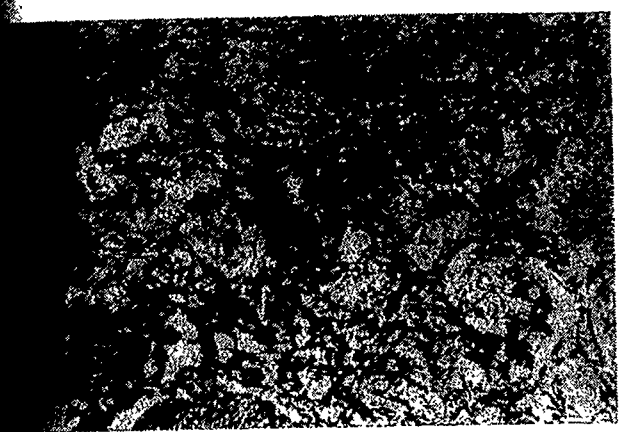

Fig. 3. Acellular collagen nodules, 240 days after the intratracheal injection of long-fibre chrysotile (silver impregnation × 100).

A progressive connective tissue response persisted in the lungs of the chrysotile B group at the termination of the experiment. Massive nodular fibrosis which involved the whole lung was frequently encountered. The nodules were mostly acellular and consisted of thick collagen bands with only a few reticular fibres interspersed (Fig. 3).

DISCUSSION

In the present investigation the relative toxicity and fibrogenicity of 5 International Reference Asbestos Samples were investigated in the lungs of rats. The physical and chemical properties of the asbestos samples were known and the mean fibre length of the samples, with one exception, was almost identical.

Macroscopically and microscopically no marked differences concerning the cellular response could be observed among the lungs injected with samples of short-fibre asbestos. The initial cellular response induced by these samples consisted of a typical foreign-body reaction. The reaction in the chrysotile B group was more severe and numerous mononuclear cells and leucocytes were mobilized, much more so than in the short-fibre asbestos groups. Similar observations have been made by other workers,[11,12] when asbestos fibres of graded lengths had been injected into the lungs of animals.

Considered critically, the present results demonstrate that short asbestos fibres from the various asbestos types are of very low toxicity and produce only peribronchiolar and perivascular reticular lesions (grade I fibrosis). These lesions were non-progressive, except when accompanied by chronic infections. Of the short-fibre asbestos samples chrysotile A appeared to be the least fibrogenic. Wagner and Skidmore[13] found that chrysotile fibres were eliminated from the lung much faster than crocidolite and amosite and this finding might explain our observation (Table III).

Long fibres of chrysotile B, in contrast with short fibres of other asbestos types, provoked a more extensive cellular reaction followed by a progressive fibrosis (grade III). The long fibres were retained in the lung for long periods, producing a state of chronic inflammation.

The incidence of infection was extremely high in the lungs of the chrysotile B group, much higher than in the short-fibre groups. When infection and inflammation persisted in the lung, fibrogenesis was markedly increased. This observation is also applicable to the short-fibre groups. The higher incidence of infection in the long-fibre group could be due to chronic irritation by these fibres which were retained in the lung tissue. Another contributing factor might be that the long fibres blocked the air passage, causing collapse of the lung followed by infection and chronic inflammation.

Although the chemical composition of the various asbestos samples used was dissimilar, no marked differences concerning their biological effects could be demonstrated histologically. From this observation it would appear that the chemical constituents of asbestos play a minor role in fibrogenesis. However, it is known that relatively large doses of some components of asbestos (i.e. silica, aluminium, iron and various trace elements) might be extremely toxic, inducing pathological changes in tissues. These elements might also indirectly enhance fibrogenesis by maintaining a chronic inflammatory state in the lung.

Although Holt, *et al.*[14] claimed that short fibres of asbestos are primarily responsible for the condition of asbestosis, our results in rats indicated that short fibres were eliminated so quickly that no permanent lung damage ensued. In contrast, the long fibres, which are retained in the lung tissue, provoked a progressive fibrosis and permanent lung damage.

From these observations it may be concluded that:

1. Long asbestos fibres are relatively more dangerous and irritating than short fibres in the lungs of rats.
2. Some chemical constituents present in asbestos may produce a chronic inflammation in lung tissue.
3. Infection and chronic inflammation enhance the fibrogenic response of lung tissue to asbestos dusts.

SUMMARY

The relative fibrogenicity of 5 standard reference asbestos samples was investigated in the lungs of female rats over a period of 240 days. The mean fibre length of amosite, antophyllite, crocidolite and chrysotile A varied between 2·0 and 3·4 μm, while chrysotile B consisted of relatively long fibres with a mean fibre length of 17·0 μm.

The samples were suspended in normal saline and administered by intratracheal injection at a dose of 50 mg asbestos per animal, except in the chrysotile B group where 25 mg dust was injected into one lung of the animal only. The results indicated that the various asbestos samples induced an initial foreign-body reaction, which appeared to be more severe in the long-fibre group (chrysotile B). The biological responses of the 4 short-fibre asbestos groups were so similar that no significant histopathological differences could be detected.

The total amount of fibrosis produced over a period of 240 days amounted to 1·63 for crocidolite, 1·52 for amosite, 1·42 for antophyllite, 1·20 for chrysotile A and 5·50 for chrysotile B. Only chrysotile B, which consisted of relatively long fibres, produced a progressive nodular fibrosis. The incidence of infection and chronic inflammation was much higher in the

long-fibre group than in the short-fibre groups.

From these observations it would appear that the relative length of the asbestos fibres is of primary importance in inducing asbestosis in the lungs of rats.

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TUMOUR-PROMOTING PROPERTIES AND METABOLISM OF 5α-CHOLEST-6-ENE AND 5α-CHOLESTA-1,3,6-TRIENE*

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Several derivatives of cholesterol such as cholest-5-en-3-one,[1] 6β-hydroperoxycholest-4-en-3-one[2] and cholest-4-ene-3,6-dione[3] produce sarcomas when injected into mice. In the present work the carcinogenic action and influence on carcinogenesis of the unsaturated steroidal hydrocarbons 5α-cholest-6-ene and 5α-cholesta-1,3,6,-triene as well as their metabolism by the mouse and rat liver microsomes are described. Both steroids have a double bond and therefore a relatively high electron density at the 6,7-position of the steroid nucleus thus resembling the so-called 'K-region' of the polycyclic aromatic hydrocarbons.

MATERIAL AND METHODS

The 5α-cholest-6-ene was synthesized by pyrolysing 5α-cholestan-6β-ylbenzoate at 0·1 mmHg pressure and 300°C for 16 hours. The product was purified by chromatography on alumina and finally recrystallized from methanol (calculated for $C_{27}H_{46}$: C = 87·56%, H = 12·44%; found: C = 87·34%, H = 12·56%; melting point and mixed melting point with a sample prepared according to Turner,[4] 81°C).

The starting material used in the synthesis of 5α-cholesta-1,3,6-triene was 2β,3α-dibromo-5α-cholestan-6β-ol. This compound was dehydrobrominated by refluxing with collidine[5] to give 5α-cholesta-1,3-dien-6β-ol and the latter product was benzoylated and pyrolysed as above. The 5α-cholesta-1,3,6-triene prepared in this way was purified by chromatography and recrystallized from methanol (calculated for $C_{27}H_{44}$: C = 88·52%, H = 11·48%; found: C = 88·36%, H = 11·46%; melting point 68°C; λ max. in alcohol 260 nm which is indicative of a homoannular diene;[6] microhydrogenation showed the presence of three double bonds).

The 5α-cholestane-6β,7α-diol and 6,7-epoxy-5α-cholestane were obtained by hydroxylation and epoxidation of 5α-cholest-6-ene as described by Fieser and Rajagopalan.[7] (Calculated for $C_{27}H_{48}O_2$ (diol): C = 80·19%, H = 11·88%; found: C = 80·13%, H = 11·81%; melting point 127°C. Calculated for $C_{27}H_{46}O$ (epoxide) C = 83·92%, H = 11·91%; found: C = 83·71%, H = 11·71%; melting point 71°C.)

Tests for Carcinogenicity

In the tests for carcinogenicity albino male mice and C57 male mice were used as experimental animals. Mice of both strains were divided into four groups and subcutaneously injected with one of the following:

(*a*) A single dose of 0·1 mg dibenz(a,h)anthracene in 0·2 ml olive oil solution.

(*b*) A single dose of a mixture of 0·1 mg dibenz(a,h)anthracene and 20 mg 5α-cholest-6-ene or 5α-cholesta-1,3,6,-triene in 0·2 ml olive oil solution.

(*c*) Ten doses at weekly intervals of 2 mg 5α-cholest-6-ene or 5α-cholesta-1,3,6-triene in 0·2 ml olive oil solution.

(*d*) Ten doses at weekly intervals of 0·2 ml olive oil.

All tumours were histologically examined.

Extraction of Metabolites from Excreta

In the metabolism experiments the steroid (30 mg) dissolved in olive oil (0·5 ml) was injected subcutaneously into albino male mice and the excreta were collected for 4 days after the injections were made. The excreta were collected daily, suspended in a little water, pooled and stored at 0°C until further investigated. The suspension of excreta was exhaustively extracted with ether. The water-phase was then acidified (pH 5·5) and incubated with β-glucuronidase (100 000 Fishman units) for 36 hours at 37°C. After this incubation period it was again continuously extracted overnight with ether. These ethereal extracts were dried on sodium sulphate and concentrated to a small volume. Controls were prepared in a similar way from the excreta obtained from mice which received only olive oil.

Experiments with Rat Liver Microsomes

Microsomes and the soluble rat liver fraction were prepared from the livers of Wistar rats (body-weight 200–250 g) as described by Booth and Boyland.[8] Each experiment was carried out with the microsomes prepared from 1 g liver to which was added nicotinamide (600 μmoles), NADP+ (1·5 μmoles), glucose-6-phosphate (30 μmoles) and 2 ml soluble rat liver fraction. The volume of the mixture was adjusted to 10 ml by the addition of 0·1 M phosphate buffer (pH 7·4) and finally a solution of the steroid (5 mg) in ethanol (0·5 ml) was added. The mixture was then incubated in air for 30 minutes at 37°C and thereafter extracted three times with ethyl acetate (6 ml portions). The extract was dried over anhydrous sodium sulphate, the solvent evaporated and the residue examined by thin-layer chromatography.

Chromatography

Thin-layer chromatography was carried out on plates

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