Comparison of effects on macrophage cultures of glass fibre, glass powder, and chrysotile asbestos

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Beck, E. G., Holt, P. F., and Manojlović, N. (1972). *Brit. J. industr. Med.*, **29**, 280-286. **Comparison of effects on macrophage cultures of glass fibre, glass powder, and chrysotile asbestos.** The effects on macrophage cultures of glass fibre, glass powder, and chrysotile asbestos are compared. Glass fibre behaves like chrysotile in producing an increase in cell membrane permeability in cultured macrophages. This is demonstrable by the increase in lactic dehydrogenase activity in the supernatant fluid. The metabolism, measured by lactate production, is not reduced as it is when quartz is phagocytosed. Glass powder behaves like the inert dust corundum, producing little change in the number of cells stained by erythrosin B and a small increase in lactate dehydrogenase activity, both being in the range of the control. There is an increase in lactate production as a result of higher metabolism due to phagocytosis. Dusts may produce two basic effects, namely a toxic effect and change in cell membrane permeability. A non-specific effect on the cell membrane due to the slow and sometimes incomplete process of ingestion of long fibres is probably a function of the morphology, particularly the length of the fibres. A primary specific effect induced by some dusts immediately follows contact with the cell membrane.

Recent investigations have shown that asbestos fibres alter the permeability of the membrane of cells cultured *in vitro*. Koshi, Hayashi, and Sakabe (1968) demonstrated an increase in the acid phosphatase released from the cells into the culture medium when rat peritoneal macrophages were incubated with asbestos. Beck (1970) and Beck, Holt, and Nasrallah (1971) found that chrysotile asbestos added to cultures of alveolar or peritoneal macrophages increased the number of cells stained with erythrosin and increased the amount of lactic dehydrogenase (LDH) released into the culture medium.

To determine whether the shape, the length or the chemical composition of the fibres is the factor influencing the permeability of the cell membrane, cultures of macrophages were incubated with two

types of fibrous material, chrysotile and glass fibre. In each case the permeability of the cell membrane was determined by estimating the percentage of cells that stained with erythrosin B and the LDH activity in the supernatant. The vitality of the cells was measured by the rate of lactate production. Controls were run with no addition of dust, with quartz powder (toxic), and with corundum powder (inert).

Materials

Chrysotile

A high-grade Rhodesian chrysotile was opened in the mill of a dust tunnel (Holt, Mills, and Young, 1964).

Glass fibre

A dust was prepared in the same way from commercially available glass fibre. The sample ranged in length from 1 to 20 μm and in diameter from 0.25 to 1.0 μm .

Glass powder

Lump glass, from which the glass fibre is made, was crushed in a rubber-lined tumbling box through which a slow current of air was passed to carry off the fine dust. The suspended dust was allowed to settle on the floor of a dust tunnel. The particle size was $< 3 \mu\text{m}$.

Quartz

Dörentztruper powdered crystal quartz, DQ 12 (specific surface $7.4 \text{ m}^2/\text{g}$, particle size $< 3 \mu\text{m}$) was used.

Corundum

The commercially available Maxalun (Al_2O_3) (specific surface $1.5 \text{ m}^2/\text{g}$, average particle size $3 \mu\text{m}$) was used.

Methods

Monolayer cultures of guinea-pig alveolar and peritoneal macrophages were prepared by the technique described by Beck (1970). The macrophages were collected in NCTC 109 medium (Difco Laboratories, Detroit, Michigan) and cooled in an ice-bath. The cells in the pool were counted in a haemocytometer and aliquots of the cells were transferred to Leighton tubes containing a coverslip. Each tube contained 4 to 5×10^6 cells. The cultures were incubated at 37°C . After one hour, when the cells had become attached to the coverslip, the supernatant was removed and the monolayer rinsed with Tyrode solution. The dust sample suspended in culture medium with or without 5% homologous serum was then added in a concentration of $75 \mu\text{g}$ of dust per 10^6 cells.

Control experiments in which quartz, corundum or no dust was added were run simultaneously with each

group of tests. LDH was determined by the method of Wieland and Pfeleiderer (1957) and lactate enzymatically by the method of Horn and Bruns (1956) 3, 6, and 20 hours after the addition of dust to the cultures. After the same intervals the cells on the coverslips in the Leighton tubes were stained with erythrosin B (Münch, Beck, and Manojlović, 1971) and examined microscopically to determine the percentage of cells stained. Cells in parallel tubes were observed by phase contrast microscopy. Photomicrographs were taken using an interference filter (540 nm).

Identical values cannot be obtained in repetitive biological tests: each value in the tables represents the average of four experiments.

Results**Peritoneal macrophages** (Table; Fig. 1)

The 'inert' control dust (corundum) had little effect on the permeability of the cell wall as measured by the uptake of acid dye and enzyme release. The metabolism, as measured by the rate of lactate production, was increased.

The addition of quartz caused a disturbance of the membrane permeability, resulting in a large increase in the enzyme liberated and increased uptake of acid dye, all the cells being stained by erythrosin 20 hours after incubation. There was a pronounced fall in lactate production.

When chrysotile was added to a culture, the number of cells stained with erythrosin was increased and more enzyme was released into the culture fluid.

TABLE
EFFECTS OF CERTAIN DUSTS ON CULTURES OF MACROPHAGES: MEAN (RANGES IN PARENTHESES)

Test	Erythrosin ¹			LDH ²			Lactate ³		
	3	6	20	3	6	20	3	6	20
<i>Peritoneal macrophages</i>									
No dust	3 (1-5)	6 (5-7)	7 (6-8)	26 (25-27)	61 (58-63)	80 (75-85)	24 (20-28)	75 (69-81)	197 (190-204)
Corundum	4 (3-5)	7 (6-8)	8 (7-9)	36 (30-42)	69 (65-73)	248 (231-265)	47 (46-48)	111 (100-122)	310 (301-319)
Quartz	85 (75-90)	91 (80-95)	100 (100)	970 (850-1090)	1090 (1000-1190)	960 (910-1010)	35 (30-40)	63 (59-67)	63 (56-70)
Chrysotile	10 (8-12)	24 (20-28)	37 (30-42)	158 (150-168)	255 (241-274)	435 (426-444)	39 (37-41)	104 (100-108)	340 (335-345)
Glass fibre	8 (7-9)	17 (15-19)	48 (45-51)	100 (93-107)	158 (149-169)	422 (411-433)	36 (34-38)	120 (109-131)	390 (381-299)
Glass powder	7 (6-8)	13 (11-15)	14 (13-15)	70 (59-81)	111 (101-121)	255 (247-263)	30 (27-33)	98 (91-115)	225 (215-235)
<i>Alveolar macrophages</i>									
No dust	7 (6-8)	10 (8-12)	11 (9-13)	70 (65-75)	70 (66-74)	75 (70-80)	24 (19-29)	39 (35-43)	94 (90-98)
Corundum	9 (7-11)	12 (9-15)	13 (10-16)	76 (70-82)	76 (71-81)	90 (87-93)	49 (46-52)	77 (74-80)	154 (148-160)
Quartz	84 (81-87)	95 (83-107)	100 (100)	650 (625-675)	690 (678-702)	660 (652-668)	19 (18-20)	28 (26-30)	29 (28-31)
Chrysotile	16 (11-21)	35 (30-40)	53 (51-55)	130 (126-134)	160 (152-168)	390 (382-398)	26 (23-29)	43 (40-46)	152 (148-156)
Glass fibre	22 (19-25)	30 (29-31)	64 (59-69)	100 (97-103)	170 (164-176)	410 (398-422)	36 (32-40)	61 (58-64)	145 (140-150)
Glass powder	14 (12-16)	15 (11-19)	16 (13-19)	75 (69-81)	80 (79-81)	100 (96-104)	63 (59-67)	83 (78-88)	124 (123-125)
<i>Alveolar macrophages (homologous serum)</i>									
No dust	7 (6-8)	10 (8-12)	11 (9-15)	70 (65-75)	75 (70-80)	80 (75-85)	45 (39-51)	71 (69-73)	121 (117-125)
Chrysotile	18 (17-19)	43 (39-47)	65 (62-68)	226 (220-232)	306 (299-313)	460 (455-463)	71 (68-74)	98 (91-105)	172 (165-179)
Glass fibre	20 (18-22)	39 (34-44)	67 (64-70)	190 (182-198)	337 (329-345)	579 (565-593)	63 (60-66)	105 (100-110)	165 (163-167)
Glass powder	11 (8-15)	12 (10-14)	14 (12-16)	80 (72-88)	113 (102-122)	132 (128-136)	51 (49-53)	89 (85-93)	155 (148-162)

1 Cells stained by erythrosin (%)

2 LDH activity in culture fluid (mU)

3 Lactate synthesis (μg)

Each experiment was run in quadruplicate. The same culture was used for all these tests.

FIG. 1. Effects of dusts on guinea-pig peritoneal macrophages: ▼ control; △ quartz; ▽ corundum; ○ chrysotile; ▲ glass fibre; ● glass powder. (A) Cells stained by erythrocin (%); (B) LDH activity (mU/5 × 10⁶ cells); (C) lactate production (μg/5 × 10⁶ cells).

There was, however, an increase in lactate production. Glass fibre also increased the permeability of the cell membrane. After 3 and 6 hours the percentage of cells stained by erythrocin and the LDH activity of the supernatant were lower than the comparable values for chrysotile but after 20 hours the values were almost the same.

Glass powder produced minimal changes in the membrane permeability but an increase in lactate production as a result of higher metabolism due to the active process of phagocytosis, comparable to corundum.

Alveolar macrophages (Table; Fig. 2)

Qualitatively, the effects of the dusts resembled the effects on peritoneal macrophages. In particular, chrysotile and glass fibre increased the percentage of alveolar macrophages stained by erythrocin to a larger extent than with peritoneal macrophages and increased the LDH activity in the supernatant; the lactate production was increased. Again, the effects of glass powder resembled those of corundum.

Figure 3 shows alveolar macrophages of a guinea-pig 20 hours after incubation with chrysotile, glass fibre and glass powder, and untreated cells. Using an interference filter (540 nm) cells stained with erythrocin appear dark. Few cells of the untreated control or the culture incubated with glass powder are stained. After incubation with fibrous dusts many more are stained. At this magnification it is

apparent that most of the stained cells have incompletely ingested long fibres.

Phase contrast microscopy at higher magnification (Fig. 4) reveals the incomplete ingestion of long chrysotile fibres and glass fibres to better advantage. The cells that incompletely incorporate fibres appear spindle-shaped like fibroblasts.

Alveolar macrophages with dust and homologous serum (Table)

The addition of 5% guinea-pig serum to the culture medium caused the macrophages to phagocytose both the fibrous and non-fibrous dusts more rapidly and in greater quantity. Chrysotile and glass fibre produced changes in the membrane permeability in the same way as when serum was absent. The percentage of cells stained by erythrocin and the LDH activity in the culture medium were raised by the serum. The cells produced more lactate, again indicating an unimpaired and elevated metabolic rate.

Comment

Two properties of cells in culture have been studied, the permeability of the cell membrane and the vitality or metabolism. The increased membrane permeability is indicated by an increase in the number of cells stained by erythrocin B and an increase in the LDH that leaks from the cell to the

FIG. 2. Effects of dusts on guinea-pig alveolar macrophages: ▼ control; △ quartz; ▽ corundum; ○ chrysotile; ▲ glass fibre; ● glass powder. (D) Cells stained by erythrosin (%); (E) LDH activity (mU/4 × 10⁶ cells); (F) lactate production (μg/4 × 10⁶ cells).

culture fluid. Change in metabolism is observed as a change in the rate of lactate synthesis.

Phagocytosis of dust particles may produce changes in the permeability of the cell membrane due to three factors:

1. Certain dusts, such as Dörentz quartz but not asbestos, increase the permeability in protein-free media within 5 minutes of contact with the cell (Münch *et al.*, 1971). The effect may be due to adsorption of membrane constituents on to the dust.

Fig. 3a

Caption for Fig. 3 overleaf

Fig. 3b

Fig. 3c

FIG. 3. Guinea-pig alveolar macrophages incubated with (a) chrysotile, (b) glass fibre, and (c) glass powder of similar composition, then stained with erythrosin B. Both fibrous dusts produced an increased membrane permeability so that many cells were stained. Although the powdered glass was ingested by the macrophages, there was little increase in membrane permeability. $\times 375$. Interference filter.

FIG. 4. Guinea-pig alveolar macrophages 120 minutes after incubation with fibrous dust: (left) glass fibre, (right) chrysotile ($150 \mu\text{g}/10^6$ cells). More than one macrophage are attached to the long fibres. Phase contrast $\times 800$.

2. A secondary reaction after some dusts have been phagocytosed is due to intracellular damage. It increases the membrane permeability and decreases the metabolism. It follows the ingestion of quartz but not corundum.

3. Long fibres of glass or asbestos increase the permeability of the cell membrane while they are being phagocytosed but they also increase the metabolism (Beck, 1970; Beck *et al.*, 1971). The ingestion of long fibres by cells is a slow process and it may be incomplete, thus the increased permeability may be sustained for many hours. Fibres with a length that exceeds the cell diameter may remain partly extracellular or several cells may become attached to one fibre, forming a structure resembling a string of beads (Fig. 4).

The evidence for the three types of change produced in the cell by dusts, as recorded in this and previous reports, may be summarized:

1. Quartz dust is rapidly cytotoxic but corundum is not. Although short fibres of asbestos and glass are phagocytosed as rapidly and completely as is quartz, this secondary toxic effect is not observed. This indicates that the secondary effect is due to composition rather than shape.

2. Long glass fibres, like long chrysotile fibres, increase the permeability of the cell membrane. Glass powder which has an identical chemical composition to the glass fibre behaves like the 'inert' dust corundum, producing no increase in permeability. Photomicrographs of cultures to which the

three dusts had been added and which were subsequently stained with erythrosin B are shown in Figure 3. The change in membrane permeability appears to be related to the process of incorporation of the fibre; the shape of the fibre and not its chemical composition must be responsible.

3. The addition of 5% serum to the culture medium increases the number of cells stained by erythrosin and the LDH value. Serum contains a phagocytosis-stimulating factor so that more fibres are phagocytosed. Protein from the serum is adsorbed by the fibres but this does not inhibit the permeability change. This suggests again that the shape of the fibres, particularly the length, rather than the nature of the surface is responsible for the increased permeability. Since more fibres are phagocytosed, there is an increase in cell metabolism and an increase in lactate production.

4. Treatment of chrysotile with dilute acid produces a still greater membrane permeability but a decrease in the lactate synthesis. The acid does not alter the morphology of the chrysotile fibres but it removes magnesium ions and leaves a surface resembling that of silica. The secondary toxic effect is now introduced. If the acid-treated chrysotile is treated with polyvinylpyridine N-oxide, the toxicity is reduced; membrane permeability now compares with that of untreated chrysotile and the metabolic rate is restored (Beck *et al.*, 1971).

5. Chrysotile that has been heated with strong acid is changed structurally and chemically; most of

the magnesium is removed and silanol surface remains (Clark and Holt, 1961). Its action on macrophages resembles that of quartz. Pretreatment with polyvinylpyridine N-oxide eliminates these cytotoxic effects (Beck, 1970).

In this connexion the findings of Szentei (1970) are relevant. She found that 0.25 mg chrysotile produced 40% haemolysis of erythrocytes *in vitro* but 8 mg glass fibre or 5.2 mg glass powder were required to produce the same degree of haemolysis. It follows that the haemolytic effect depends on the chemical composition rather than the morphology or length of the fibres. It is probable that the monolayer technique of cell culture has some disadvantages when fibrous dusts are investigated. Because the cells lie in a plane, some fibres do not come into contact with cells but form a network in the medium above the monolayer, an effect that is being investigated.

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