Review Article

Biological Effects of Asbestos Fibers on Human Cells *In Vitro*—Especially on Lymphocytes and Neutrophils

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Abstract: Biological effects of asbestos fibers were reviewed in relation to the polyclonal activation of human lymphocytes and to the release of free radicals from human neutrophils *in vitro*. Chrysotile, crocidolite, and amosite asbestos activate CD4⁺ T lymphocytes polyclonally, followed by activation-induced cell death (a type of apoptosis). The activation is HLA class II dependent, and certain V β repertoire, e.g. V β 5.3, are detected among the fractionated T cells with a high Ca⁺⁺ level that had been stimulated by asbestos fibers. These observations support the possibility that asbestos acts as a superantigen, and that asbestos stimulate lymphocytes repeatedly *in vivo*. It has been reported that asbestos-induced cytotoxicity can be suppressed by the scavengers of superoxide or hydroxyl radical. Some of these scavengers such as dimethylsulfoxide (DMSO) or retinoic acid are known as inducers of cell differentiation. The biological functions of DMSO for cell differentiation of HL-60 cells to neutrophils are suppressed by co-culturing of crocidolite asbestos, because DMSO reacts with the hydroxyl radical released after the stimulation with crocidolite and spent itself. Superoxide dismutase (SOD) inhibited the effects of crocidolite, reacting rapidly with $\cdot\text{O}_2$ before the secondary release of $\cdot\text{OH}$. It seems to be probable that asbestos fibers, especially crocidolite, suppress the tissue cell differentiation by releasing free radicals and by wasting inducers of cell differentiation as radical scavengers.

Key words: Asbestos, Chrysotile, Crocidolite, Amosite, Lymphocytes, Activation, Apoptosis, Free radicals

Introduction

Asbestos is a term that is applied to a group of naturally occurring, hydrated mineral silicates that are separable into fibers. Asbestos fibers are known to be associated with a variety of human diseases including lung fibrosis¹⁾, lung cancer^{2,3)}, malignant mesothelioma³⁾, malignant lymphoma⁴⁾, and systemic immunological disorders as systemic sclerosis (SSc)⁵⁾. Subjects with occupational exposure to asbestos have been reported to demonstrate increased CD4⁺/CD8⁺ T cell ratios⁶⁾. Bozelka *et al.*⁷⁾ reported that the addition of asbestos fibers to human peripheral blood lymphocytes, stimulated with concanavalin A (Con A) or phytohaemagglutinin (PHA), resulted in a significant increase in the early mitogenic

response. To the contrary, Barbers *et al.*⁸⁾ described that the late mitogenic response in human peripheral lymphocytes to PHA was depressed by asbestos fibers.

We designed to examine the involvement of asbestos fibers in the proliferation of various kinds of human cells especially lymphocytes. The observations of Bozelka *et al.*⁷⁾ coincided with our results that detected the polyclonal activation of human T lymphocytes by asbestos fibers as so-called “superantigens”. In addition, the results of Barbers *et al.* could be explained in accordance with our observations that asbestos fibers induce the activation-induced cell death after polyclonal activation of T cells. The activation of PHA was strengthened by the co-culturing with asbestos fibers and followed by elevated apoptosis of T cells. Therefore the

incorporation of ^3H -thymidine for DNA synthesis decreases in the late stage (in 6–7 days) of incubation. Details are mentioned in the following chapter “Effects on the cell proliferation and activation”.

In other works, we analysed the effects of silica in the induction of several kinds of autoantibodies or in the levels of soluble Fas (sFas) or soluble Fas ligand (sFasL) in the sera from the patients with silicosis, and detected no correlation between the severity of lung fibrosis and that of the immunological disorders among the patients (data not shown), although the patients with asbestosis have not been analysed.

The release of free radicals plays an important role in the cytotoxicity and tumor promotion caused by asbestos fibers. Some studies have shown that asbestos-induced cytotoxicity can be suppressed by the scavengers of superoxide or hydroxyl radical^{9, 10}. Some of these radical scavengers, e.g. DMSO or retinoic acid, play as inducers of cell differentiation at the same time. The authors intended to know whether the biological function of inducers of cell differentiation could be influenced by asbestos fibers which cause the release of free radicals. The details of the results are mentioned in the chapter “Release of free radicals”.

Effects on the Cell Proliferation and Activation

Several types of cells were incubated with asbestos fibers *in vitro*, and the biological effects of asbestos fibers were analysed. Asbestos fibers (chrysotile, crocidolite, amosite, and anthophyllite), the International Union against Cancer (IARC) standard reference sample were kindly provided to us by the National Center for Occupational Health, South Africa. The fibers were sterilized by heating at 180°C for 1h, and suspended in the culture medium. The exposure of cells to asbestos fibers were performed at a final concentration of 50 or 100 $\mu\text{g}/\text{ml}$ of fibers, in which conditions no acute cytotoxicity was detected.

Effects of asbestos fibers on the proliferation of human cell lines

Differences in the biological effects on human cells were detected between types of asbestos fibers. Chrysotile and crocidolite acted sometimes mitogenically and sometimes cytotoxically according to the type of cells. Chrysotile and crocidolite were mitogenic to Raji, Daudi and SB cell lines which possess B lymphocyte surface markers and no cytoplasmic immunoglobulin. However, no effect was observed on MOLT-4 T cell line, K-562 erythromyeloid cell line, and RPMI 1788 B cell line with cytoplasmic IgM.

Moreover, chrysotile and crocidolite were significantly cytotoxic to HEL-299 fibroblasts or J-111 monocytic cells. Amosite showed milder biological effects on the proliferation of human cell lines than those of chrysotile or crocidolite (Fig. 1)¹¹.

Effects of chrysotile on CD4 or CD8 expression in PHA activated cells

After stimulation of lymphocytes, the temporal decrease in CD4 or CD8 surface markers in T lymphocytes and its recovery in a short time is thought to imply the evidence of lymphocyte activation^{12–14}. During a 6–12 h stimulation of peripheral blood mononuclear cells (PBMC) with phytohaemagglutinin (PHA), the surface CD4 antigen declined remarkably, but returned to the original level in 24 h. Cell viability was more than 90% constantly, determined by flow cytometric analysis and the trypan blue dye exclusion test. When chrysotile was present in the culture system, the expression of CD4 was significantly lower compared to cells stimulated with PHA alone ($P < 0.01$) and returned to the original level in 24 h without any cytotoxic effect from chrysotile (Fig. 2). The modulation of markers was most prominent in 12 h after stimulation generally¹². No significant difference in modulation of CD8 antigen was observed between cells activated with PHA alone or with PHA and chrysotile. No difference in cell viability was observed between PBMC cultured with or without chrysotile asbestos.

Activation of human CD4⁺ CD45RA⁺ T cells by asbestos fibers in vitro

During 6 to 12 h incubation of PBMC with chrysotile, crocidolite or anthophyllite, the surface CD4 antigen declined significantly, but returned to the original level within 24 h. When amosite was added to the culture system, the modulation of CD4 was not so prominent (Fig. 3)¹⁷.

As in the case of CD4, cell surface CD45RA expression in PBMC was downregulated after incubation with chrysotile *in vitro*. The expression of CD45RA declined significantly within 12 h after incubation with chrysotile, crocidolite, anthophyllite and amosite respectively ($p < 0.05$). The modulation of CD45RA continued for a long time, and was also detected 72 h after incubation. The CD45 family consists of isoforms, which are generated by the alternative splicing of exons encoded by a single gene^{15, 16}. Freshly isolated CD4⁺ CD45RA⁺ cells are considered to be naive cells. CD4⁺ CD45RA⁺ cells express both CD45RO and CD29.

An increased percentage of IL-2R positive cells was also indicative of the activation of PBMC by chrysotile asbestos

Fig. 1. Effects of asbestos fibers on the cell proliferation.

Human cell lines; (a) Raji, (b) Daudi cells, (c) RPMI 1788, (d) SB, (e) MOLT-4, (f) K-562, (g) HEL-299, and (h) J-111. Cell suspension (5×10^5 cells/ml) was incubated with asbestos at 37°C for 48 h, and cell count was calculated. Chrysotile= — ; crocidolite= — ; amosite= — ¹¹⁾.

Fig. 2. Modulation of cell surface phenotypes CD4 and CD8 after activation of PBMC with $10 \mu\text{g/ml}$ PHA (—) or both PHA and $50 \mu\text{g/ml}$ chrysotile (—). Mean $\pm$ S.D.; $n=10^{12}$.

Fig. 3. Modulation of cell surface CD4 and CD45RA after incubation of PBMC with 50 µg/ml crocidolite (a), anthophyllite (b), and amosite (c) asbestos fibres.

— CD4⁺ CD45⁺ cells; - - CD4⁺ cells; ... CD45RA⁺ cells. Calculation of the index was based on the control cells incubated without asbestos fibres. Mean ± SD n=6⁽¹⁷⁾.

(data not shown)⁽¹⁷⁾.

Cell viability was more than 90% constantly (data not shown), determined by flow cytometric analysis with fluorescein diacetate (FDA) and propidium iodide (PI) double-staining method (Alive cells are stained with FDA and dead cells are PI positive.). No difference in cell viability was observed between PBMC cultured with or without asbestos fibers.

These observations also suggest that chrysotile, crocidolite, anthophyllite and amosite stimulate T lymphocytes *in vitro*⁽¹⁸⁾.

Intracellular Ca⁺⁺ level in PBMC after stimulation

It has been known that intracellular Ca⁺⁺ levels elevate after the stimulation of lymphocytes. Therefore, Ca⁺⁺ levels were analysed to detect the stimulation of lymphocytes by asbestos fibers. PBMC were pre-incubated with Ca indicator Fluo3-AM (10 µg/ml) for 30 min at 37°C, washed in Hanks' solution, then incubated with 50 µg/ml chrysotile for 0, 1, 3, and 10 min. The lymphocyte fraction was gated and analysed flow cytometrically. Then, a high incidence of stimulated cells with an increased intracellular Ca⁺⁺ level was observed. The elevated intracellular Ca⁺⁺ level could still be detected after 30 min of incubation (Fig. 4)⁽¹⁸⁾.

Vβ repertoire of stimulated lymphocytes

We investigated whether chrysotile acts on human lymphocytes as a superantigen that activates all T lymphocytes bearing the appropriate T cell receptor Vβ repertoire. Lymphocyte fraction with elevated intracellular Ca⁺⁺ was gated flow cytometrically and the Vβ repertoire of chrysotile-stimulated lymphocytes from healthy individuals was investigated. A significant increase in the expression of Vβ 5.3 or Vβ 6.7 was found in several samples. Then, the fractions having a high Ca⁺⁺ level were collected and analyzed for the repertoire. The repertoire was not restricted, and TcR Vβ 6.7 increased predominantly in case I, and Vβ 5.3 in case II and III, whereas such a predominant increase was not observed in the lymphocytes stimulated with PHA (Data not shown)⁽⁹⁾. These results suggest that chrysotile activates human lymphocytes as a superantigen, which means the possible activation of autoreactive T lymphocytes by asbestos fibers.

IL-2 release from PBMC after stimulation

The increased secretion of IL-2 from the lymphocytes can be observed after lymphocyte stimulation. A bioassay of IL-2 secretion from PBMC was performed using an IL-2-dependent mouse cytotoxic T-cell line CTLL-2. PBMC were incubated with or without chrysotile for 48 hr. The

Fig. 4. Intracellular Ca^{++} level in human peripheral blood mononuclear cells (PBMC) after incubation with chrysotile.

PBMC were pre-incubated with Ca indicator Fluo3-AM (10 $\mu\text{g}/\text{ml}$) for 30 min at 37°C , washed in Hanks' solution, then incubated with 50 $\mu\text{g}/\text{ml}$ chrysotile for 0, 1, 3, and 10 min. The cells were analysed flow cytometrically¹⁸⁾.

culture supernate was used for the bioassay. The release of IL-2 was significantly higher in PBMC incubated with chrysotile than in control cells ($P < 0.05$, Table 1)¹⁹⁾.

To examine the requirement of major histocompatibility complex (MHC) class II expression for stimulation of PBMC by chrysotile, anti-HLA DP/DR monoclonal antibody and complement were used to deplete the cells expressing HLA class II. There was no difference in IL-2 secretion between cells with and without addition of chrysotile after depletion of HLA class II. Thus MHC class II products were necessary for the response of human T cells to asbestos fibers. The number of IL-2 receptor (IL-2R) positive cells also increased ($P < 0.05$) in PBMC incubated with chrysotile. A high incidence of IL-2R was noted in CD4^+ cells but not in CD8^+ cells (data not shown)¹⁹⁾.

Activation-induced cell death

The superantigenicity of chrysotile was mentioned above.

Table 1. Bioassay of IL-2 released in response to chrysotile stimulation

Culture supernate from	Incorporation of [^3H]TdR (d.p.m.)	
PBMC	6424 ± 203	] $p < 0.05$
PBMC + chrysotile	$10,108 \pm 4205$	
PBMC + anti-HLA DR/DP + complement	3196 ± 1482	
PBMC + anti-HLA DR/DP + complement + chrysotile	3317 ± 1557	
Cell-free culture medium	4532 ± 2257	
Cell-free culture medium + chrysotile	4266 ± 1185	

PBMC were incubated with or without chrysotile (100 $\mu\text{g}/\text{ml}$) for 48 hr. Some PBMC were pretreated with anti-HLA DP/DR mAb and fresh human plasma as the source of complement for 1 hr, washed with PBS and incubated with or without chrysotile (100 $\mu\text{g}/\text{ml}$) for 48 hr. The culture supernate was centrifuged at 3000 rpm for 5 min and used for the bioassay. IL-2-dependent CTLL-2 cells ($2 \times 10^5/\text{well}$) were incubated with the culture supernate of PBMC (diluted 1:1 with RPMI-1640 medium supplemented with 15% FCS for 48 hr. [^3H]thymidine (1 $\mu\text{Ci}/\text{well}$) was added to each well 16 hr before cell harvest. Statistical significance was analysed by the Wilcoxon test 23). Mean $\pm$ SD ($n=5$)¹⁹⁾.

Fig. 5. (a) Flow cytometrical quantitation of FITC-dUTP incorporated at the 3'-hydroxyl ends of the fragmented DNA according to the TUNEL assay; **(b)** Calculation of apoptotic cells as in (a). The cells were incubated with or without chrysotile (50 $\mu\text{g}/\text{ml}$) for 0–6 days. White, chrysotile (-); Black, chrysotile (+)²¹⁾.

Superantigens such as staphylococcal enterotoxin B are known to cause activation-induced cell death (a type of apoptosis) in human T cells²⁰⁾. Investigation was performed to know whether chrysotile induces T cell apoptosis after stimulation as a superantigen, using TUNEL (TdT mediated dUTP-biotin nick end Labelling) assay. PBMC were incubated with chrysotile for 0–6 days at 37°C. The In Situ Cell Detection Kit for the TUNEL method was used to detect apoptotic cells, using flow cytometrical quantitation of FITC-dUTP incorporated at the 3'-hydroxyl ends of the fragmented DNA. As shown in Fig. 5, flow cytometrical analysis demonstrated a multitude of apoptosis in the cells incubated with chrysotile. The percentage of TUNEL positive cells was calculated from day 0 to day 6 of incubation. The maximum incidence of TUNEL positive cells was observed on day 4, and could be detected at a high level until day 6 of incubation. Small-sized cells, which imply the apoptotic cells, also increased on day 4 of the incubation (Fig. 6-a, 6-b)²¹⁾.

The percentage of monocytes contained among PBMC was calculated with an anti-CD14 monoclonal antibody. On day 4, when the apoptotic cells were most prominent, CD14⁺

Fig. 6. (a) Flow cytometrical analysis for cell size of PBMC incubated with or without chrysotile (50 $\mu\text{g}/\text{ml}$).

The cells smaller than 50 in historical scale were considered apoptotic cells; **(b)** Small sized cells incubated with or without chrysotile for 1–6 days. The index was based on the control cells. **(c)** Fas expression in PBMC incubated with or without chrysotile for 1–6 days. white, chrysotile (-); Gray, chrysotile (+)²¹⁾.

cells was less than 2% in total cells. These results suggest that the apoptotic cells on day 4 mainly consist of lymphocytes (Data not shown).

Moreover, Fas expression from day 1 to day 3 was 25% higher in the cells stimulated with chrysotile than in the control cells, however decreased promptly on day 4 to 80% of that in the control cells (Fig. 6-c), suggesting that the Fas molecule participates in the apoptosis of PBMC after stimulation with chrysotile²¹.

Based on the results, chrysotile asbestos causes activation-induced cell death through Fas-FasL system in human peripheral blood lymphocytes. In the individuals exposed to asbestos fibers for a long time, asbestos may play a role to induce repeated activation and then activation-induced cell death of lymphocytes.

Release of Free Radicals

According to Hansen and Mossman⁹, the exposure of rat macrophages to asbestos causes a significant increase in superoxide release from the cells. Asbestos-induced toxicity to tracheal epithelial cells, lung fibroblasts, and alveolar macrophages *in vitro* can be inhibited by both superoxide dismutase (SOD) and the scavengers of the hydroxyl radical²². Mossman also reported that synthetic analogs of vitamin A, a radical scavenger, inhibited the hyperplasia and squamous metaplasia of tracheal epithelium²³. Some of radical scavengers, e.g. DMSO or retinoic acid induce the differentiation of cells. We analysed for the biological function of inducers for cell differentiation could be affected by asbestos fibers.

The human promyelocytic cell line HL-60 constitutes a useful system for the study of cell differentiation, since it can be induced to differentiate to mature myeloid cells with DMSO²⁴ or to macrophages with retinoic acid²⁵. Increased protein kinase C (PKC) activity²⁶, and marked diminution of c-myc gene expression²⁷ during HL-60 cell differentiation induced by DMSO and/or retinoic acid have been reported. Several kinds of surface antigens, such as CR1, CR3 and FcR, become detectable with the differentiation of HL-60 cells²⁸.

In our studies, PKC activity, c-myc protein expression, and cell surface CR3 expression were used as the markers of cell differentiation. We observed that the induction of cell differentiation in HL-60 cells by DMSO was suppressed when asbestos, especially crocidolite, was added to the culture system, but the effect disappeared after coculturing with SOD²⁹.

Fig. 7. EPR spectra studied with HL-60 cells.

HL-60 cells were induced to differentiate to neutrophils with 1.25% DMSO for 10 days, and suspended in Hanks' solution. A mixture of the cell suspension (1×10^7 cells/ml), stimulus (100–500 ng/ml PMA or 50 µg/ml crocidolite), and DMPO (1%, 0.09 M) was transferred to a EPR cuvette. Each scan took 2 min. (A) EPR spectra with PMA: (1) Before addition of PMA; (2) 10 min after addition of PMA (100 µg/ml); (3) PMA and SOD (100 U/ml); (4) PMA and DMSO (1.25%) to cell suspension (1×10^7 /ml) supplemented with 1% DMPO. (B) EPR spectra with crocidolite: (1) Before stimulation; (2) 1 min; (3) 13 min; (4) 37 min after addition of crocidolite (50 µg/ml) to cell suspension (1×10^7 /ml) supplemented with 1% DMPO. (C) The effect of radical scavengers of EPR spectra: (1) Before stimulation; 60 min after addition of (2) crocidolite (50 µg/ml); (3) crocidolite and DMSO (1.25%); (4) crocidolite and SOD (100 units/ml) to cell suspension (1×10^7 /ml) supplemented with 1% DMPO. (D) Cell free controls: Hanks' solution containing 1% DMPO (1); Hanks' solution containing 1% DMPO and 50 µg/ml crocidolite (2)²⁹.

Electron paramagnetic resonance (EPR) spectrometry

For the detection of $\cdot\text{O}_2^-$, $\cdot\text{OH}$ or $\cdot\text{CH}_3$ the electron paramagnetic resonance (EPR) spintrapping technique was used by employing 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) as a radical trapping reagent³⁰. HL-60 cells were incubated to induce to neutrophils with 1.25% DMSO for

Fig. 8. Flow cytometrical analysis of c-myc protein positive cells.

HL-60 cells were fixed with cold ethanol containing 1% acetic acid for 5 min, washed with 0.5% Tween 20-PBS, and stained with anti-c-myc protein mAb, biotinylated goat antimouse IgG, and FITC-avidin at 4°C. The percentage of positive cells was calculated by flow cytometry. 1) unstained, 2) non-specifically stained, 3) control HL-60 cells, 4) HL-60 cells incubated with 1.25% DMSO, 5) HL-60 cells incubated with 1.25% DMSO and 50 µg/ml of crocidolite for 4 days²⁹.

10 days, and suspended in hanks' solution. A mixture of the cell suspension (1×10^7 cells/ml), stimulus (100–500 ng/ml PMA or 50 µg/ml crocidolite), and DMPO (1%, 0.09 M) was transferred to EPR cuvette. For detection of $\cdot\text{CH}_3$, a mixture of the cell suspension, crocidolite, DMPO and DMSO (1.25%) was used for the experiments. Spectrometry was performed using an EPR spectrophotometer²⁹.

The DMPO-OH signal was detected after the addition of PMA, and was significantly reduced in the presence of SOD or DMSO (Fig. 7A). After stimulation with crocidolite asbestos alone a DMPO-OH signal was observed (Fig. 7B), and it was reduced with DMSO, one of the $\cdot\text{OH}$ scavengers (Fig. 7C). The DMPO-OH signal was markedly decreased in the presence of SOD, a $\cdot\text{O}_2$ scavenger (Fig. 1C). Two spin-trapped adducts (DMPO-OH and DMPO- CH_3) were detected after incubation of DMSO with the crocidolite and DMPO mixture of cells (Data not shown); this fact suggests the conversion of DMSO to CH_4 after the reaction with $\cdot\text{OH}$. Generation of the hydroxyl radical was also detected during the stimulation of HL-60 cells with crocidolite. The appearance of $\cdot\text{OH}$ declines significantly after the addition of DMSO, which is a potent scavenger of $\cdot\text{OH}$ ³¹. The generation of the hydroxyl radical is also inhibited by the addition of the $\cdot\text{O}_2$ scavenger SOD, suggesting that $\cdot\text{O}_2$ is the initial product of the respiratory burst reaction by crocidolite, as stated by Ueno *et al.*²⁸.

c-myc protein expression

The expression of c-myc protein is characterized in HL-

60 cells, and the expression decreases with the differentiation of HL-60 cells to neutrophils. Therefore, the effects of asbestos fibers on the cell differentiation by DMSO was analysed using c-myc protein expression as a marker protein.

HL-60 cells were fixed with cold ethanol containing 1% acetic acid for 5 min, washed with 0.5% Tween 20-PBS, and stained with anti-c-myc protein mAb, biotinylated goat anti-mouse IgG, and FITC-avidin at 4°C. The percentage of positive cells was calculated by flow cytometry. More than 90% of HL-60 cells expressed c-myc protein originally. After the induction of differentiation with 1.25% DMSO for 4 days, the percentage of c-myc protein positive cells was about 65% ($P < 0.05$) of total cells. When crocidolite (50 µg/ml) was added to the system, c-myc expression increased to about 88% of total cells (Fig. 8)²⁹.

These results demonstrate that the cell differentiation of HL-60 cells by DMSO was inhibited when asbestos fibers were co-cultured. The inhibition of cell differentiation in normal human tissues is known to induce malignant transformation of cells.

Conclusions

Silica and silicate including asbestos fibers have been known to possess "adjuvant effects". The findings of polyclonal human T cell activation by silicate, which were summarized in this review article, coincide with the idea of "adjuvant effects". Polyclonal activation of lymphocyte, so-called superantigenicity, is known in other inorganic

Fig. 9. Schematic lineage of the biological effects of asbestos fibers on human cells.

substances as mercuric chloride or gold thiomalate^{32,33}). Since superantigens are considered to play an important role in the pathogenesis of autoimmune diseases, the findings mentioned above might contribute to the analysis for the pathogenesis of immunological disorders accompanied by silicosis or asbestosis.

The release of radicals plays an important roles in the cytotoxicity and tumor promotion caused by asbestos fibers⁹. From the observations mentioned in this review, it is suggested that asbestos fibers inhibit the function of inducers which stimulate immature cells to differentiate, because such inducers are frequently radical scavengers and spent themselves to react with free radicals caused by asbestos fibers.

The relationship between the factors mentioned in this review was summarized in Fig. 9 schematically.

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