# Mineral fiber-mediated activation of phosphoinositide-specific phospholipase c in human bronchoalveolar carcinoma-derived alveolar epithelial A549 cells

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**Abstract.** Given the role of phosphoinositide-specific phospholipase C (PLC) isozymes in the control of cell growth and differentiation we were prompted to analyze the expression of some of these PLC in human bronchoalveolar carcinoma-derived alveolar epithelial A549 cells. The effects of several fluoro-edenite fibers were compared with those of tremolite, a member of the calcic amphibole group of asbestos that originates from Calabria (Italy), and crocidolite, that, due to its high toxicity, is one of the most studied asbestos amphiboles. Our data show an increased expression of both PLC ß1 and PLC γ1 in A549 cells treated with asbestos-like fibers, hinting at a role of PLC signalling in those cancerous cells.

## Introduction

Phosphoinositide-specific phospholipase C (PLC) isozymes, found in eukaryotes, comprise a related group of proteins that cleave the polar head group from inositol phospholipids. Under the control of cell surface receptors, these enzymes hydrolyze the highly phosphorylated lipid phosphatidylinositol 4,5-bisphosphate [PI(4,5)$P_2$], generating two intracellular products, inositol 1,4,5-trisphosphate ($InsP_3$), a universal calcium-mobilizing second messenger, and diacylglycerol (DAG), an activator of protein kinase C (PKC) (1). Calcium mobilization and protein kinase C activation are essential for many cellular functions, because they mediate such processes as secretion, cell growth, differentiation, and proliferation (2). Of note, PKC has been reported to activate a reduced nicotinamide adenine dinucleotide phosphate oxidase on plasma membrane, which produces superoxide anions from oxygen (3). Eleven distinct isoforms of PLC, which are grouped into four subfamilies (ß, γ, δ, ε), have been identified in mammals, and several forms have been molecularly cloned (4). All PLC isoforms contain X and Y domains, that form the catalytic core, as well as various combinations of regulatory domains common to many other signalling proteins. The presence of distinct regulatory domains in PLC isoforms renders them susceptible to different mode of activation (4).

It has been demonstrated that asbestos fibers, containing varying amounts of iron (5), can catalyze the Haber-Weiss reaction, generating the reactive oxygen species (ROS) $^{\bullet}OH$. Many of the biological effects of asbestos are postulated to derive from its ability to generate reactive oxygen species (6). The exact mechanism of the oxygen radicals release is still unclear. However, it is known that asbestos stimulates ROS generation by interaction with cellular membranes or through protein tyrosine kinase (PTK), PLC, and PKC pathway activation in a dose-response manner (7).

Fluoro-edenite ($NaCa_2Mg_5Si_7AlO_{22}F_2$) is a new fibrous mineral species similar in size and morphology to some amphibolic asbestos fibers (tremolite, actinolite, antophyllite) (8) and it is known that its exposure may cause chronic inflammation and, mostly, mesothelioma cancer (9,10). Our previous studies show that fluoro-edenite induces functional modifications in human lung fibroblasts and alveolar epithelial cells with consequent DNA damage (11).

Cytokines and growth factors derived from alveolar macrophages and lung epithelial cells are strongly implicated as

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mediators of asbestos-induced pathophysiological responses (12). Among cytokines, it has been demonstrated that IL-1ß may act through the PCL pathway to activate PKC (13). On these bases, we thought to investigate if fluoro-edenite exposure may induce cytokine increase related to PLC activation. To this purpose human lung epithelial A549 cells, used as model for studying interaction between environmental particulates/fibers and human respiratory epithelium (14) were treated with two different types of fluoro-edenite fibers. The effects of several fluoro-edenite fibers were compared with those of tremolite, a member of the calcic amphibole group of asbestos that originates from Calabria (Italy), and crocidolite, that, due to its high toxicity, is one of the most studied asbestos amphiboles.

## Materials and methods

*Cell cultures*. A549, which are human bronchoalveolar carcinoma-derived cells with some features characteristic of alveolar epithelial type II cells, were obtained from American Type Culture Collection (Rockville, MD, USA) and routinely maintained in Dulbecco's modified Eagle's medium (DMEM) (InVitrogen, Italy) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 25 $\mu$g/ml fungizone and incubated at 37˚C in a humidified, 95% air/5% $CO_2$ atmosphere. For experiments the cells were trypsinized, counted in a haemocytometer, and plated either in 12-well plates (for fluorescence experiments) or in 100 mm Petri-dishes (for Western blot, and ELISA tests).

Tremolite, crocidolite and fluoro-edenite particles were suspended in culture medium and added to the cell cultures by a micro-syringe in amounts corresponding to final concentrations of 50 $\mu$g/ml (10.6 $\mu$g/cm$^2$). Cells were then incubated for 48 h.

*Asbestos fibers*. The samples of amphibolic fluoro-edenite, tremolite, and crocidolite (OSHA standard) fibers were kindly provided by Professor A. Gianfagna, Department of Earth Sciences, University 'La Sapienza', Rome, Italy. Fluoro-edenite fibers were collected in the area of Biancavilla (Sicily), and were subsequently analysed by microanalytical spectroscopic methods (SEM-EDS, TEM-EDS, XRD, FT-IR, Mössbauer and Raman). Comparison of these samples with the standards of prismatic fluoro-edenite, which originated from the same volcanic materials of that area and were previously described (8), revealed identical composition and crystal structure. Typology of some fibers also showed winchitic and richteritic compositions due to the minor differences in chemical composition. A method for enrichment and purification method for amphibolic fibers, which removed the components of the rock matrix, was developed. A gravimetric sedimentation method was used to concentrate the fine material, exploiting both different mineral densities and their relevant morphologies. This method allowed obtaining a fibrous material with up to 95% of the amphibolic fiber content. Three fractions of different types of fibrous fluoro-edenite were obtained and used in this study and were labelled as fluoro-edenite 19 and fluoro-edenite 27. These two forms differed in their iron content and the $Fe^{2+}/Fe^{3+}$ ratio. Form 27 had about 70% of $Fe^{3+}$, whereas form 19 had 50% $Fe^{3+}$ and a lower total content of iron than form 27. From the crystal structure of crocidolite asbestos, it is known that iron in this mineral is distributed among three octahedral sites, namely $M_1$, $M_2$, and $M_3$. According to Mössbauer spectra of the International Agency for Research on Cancer reference sample of UICC crocidolite, the $M_1$ sites are populated by both ferric (14%) and ferrous (29%) ions, while the $M_2$ and $M_3$ sites are exclusively occupied by ferric (40%) and ferrous (17%) ions, respectively (15). The unit structure of tremolite is represented as $[Ca_2Mg_2[SiO_{22}](OH)_2]$, with a total amount of iron of 2.98% and the relation between ferrous ferrous and ferrous ferric of 83% and 17% respectively (Mössbauer). All fibers had an average length and diameter of 30 $\mu$m and 5 $\mu$m, respectively.

*Western blotting*. The expression of ß1, γ1 phosphoinositide-specific phospholipase C (PLC) was evaluated by Western blot analysis. Briefly, the untreated and treated cells were washed twice with ice-cold PBS and collected with lysing buffer (50 mM Tris-HCl plus 20 mM EDTA and 0.5% SDS, pH 7.5). After cooling for 30 min at 0˚C, cells were homogenized and centrifuged at 13,000 x g for 15 min. Thirty micrograms of total protein, present in the supernatant, were loaded on each lane and separated by 10% Novex Bis-Tris gel electrophoresis (NuPAGE, Invitrogen) according to published methods (16). Proteins were then transferred to nitrocellulose membranes (Invitrogen) in a wet system. The transfer of proteins was verified by staining the nitrocellulose membranes with Ponceau S and the Novex Bis-Tris gel with Brillant blue R. Membranes were blocked in Tris buffered saline containing 0.01% Tween-20 (TBST) and 10% non-fat dry milk at 4˚C overnight. Primary mouse anti-PLC ß1 (D-8), PLC γ1 (E-12) (Santa Cruz Biotechnology, Santa Cruz, CA) monoclonal antibodies and rabbit polyclonal α-tubulin antibody (Sigma, Milan, Italy) were diluted (1:200) in TBST and membranes incubated for 2 h at room temperature. Antibodies were detected with horseradish peroxidase-conjugated secondary antibody using the enhanced chemiluminescence detection Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL). Bands were measured densitometrically and their relative density calculated based on the density of the α-tubulin bands in each sample. Values were expressed as arbitrary densitometric units corresponding to signal intensity.

*Immunocytochemistry*. Immunostaining for PLC was performed on untreated control or treated cell cultures. Briefly, cells were fixed with 4% phosphate-buffered paraformaldehyde, for 20 min at room temperature; quenching was performed with a solution of 2% hydrogen peroxide ($H_2O_2$) and 10% methanol in phosphate-buffer saline (PBS), for 1 min. Cells were then blocked with PBS containing 5% normal goat serum (NGS) and 0.3% Triton X-100 and incubated overnight with primary mouse monoclonal anti-PLC ß1, anti-PLC γ1 (1:200 dilution, Santa Cruz Biotechnology) antibodies. Fluoresceine isothiocyanate (FITC) labelled anti-mouse antibody (1:100, Santa Cruz Biotechnology) was used to visualize the anti-PLC ß1 and anti-PLC γ1. n 4',6-diamidino-2-phenylindole (DAPI)

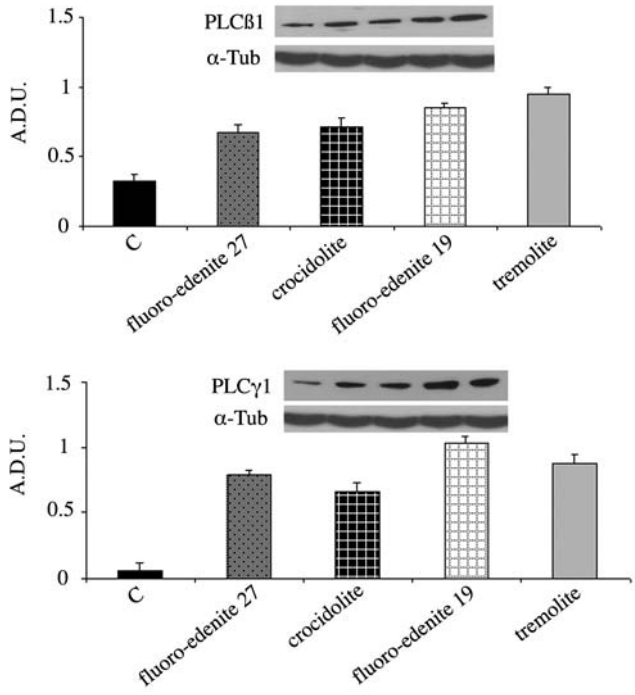


Figure 1. ß1, γ1 phosphoinositide-specific phospholipase C (PLC) expression, in treated A549 cells, evaluated by Western blot analysis.

counterstains nuclei. Coverslips were washed and mounted in PBS/glycerol (50:50) and placed on glass microscope slides. In all instances negative controls without primary antibody were performed. Coverslips were analysed on a Reichert Jung fluorescent microscope and positive cells were counted over the entire coverslips.

*Assay of cytokines in cell supernatants*. All pro-inflammatory cytokine concentrations, such as interleukin-1ß (IL-1ß), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) were measured by a specific sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems). All assays were performed as specified by the manufactures of the respective kits. The minimum detectable dose of IL-6 was typically <0.70 pg/ml, of IL-1ß <1.0 pg/ml, and of TNF-α ranged from 0.5 to 5.5 pg/ml.

*Statistical analysis*. Statistical analysis was performed with statistical software package SYSTAT, version 9 (Systat Inc., Evanston IL, USA). Each result was calculated as a mean value + standard error (SEM). Evaluation of the statistical significances between paired and unpaired values was performed by Student's t-test. Values of $p<0.05$ were considered to represent statistical significance.

## Results

Four asbestos fibers (fluoro-edenite 27, and 19, tremolite and crocidolite) were examined to evaluate what types of PLC (ß1, γ1), are involved in the answer of lung epithelial cells following treatment with each fiber using Western blotting and immunocytochemistry. Compared to the untreated cells, all fibers induced PLC expression in A549 cell following exposure to 50 $\mu$g/ml for 48 h although the level of expression induced by each fiber was different. Among the four fibers, at the end of incubation fluoro-edenite 19 showed the highest level of expression of PLC ß1, and γ1 even if a peak level for PLC ß1 was observed in tremolite-treated cells (Fig. 1). In fluoro-edenite 27 the value of expression was similar for PLC γ1, and δ1. Moreover, densitometric analysis of blots indicated that crocidolite induced the lower expression of PLC compared to the other fibers.

These results were confirmed by immunocytochemistry assays and several representative results are shown in Figs. 2 and 3. It is possible observe that in fiber-treated cells PLC γ1 appeared localized mainly in the cytoplasm and its presence was detectable also in the nucleus. In addition, the immunostaining of A549 demonstrated that all fibers were able to promote the formation of giant multinucleated cells, although the phenomenon was greater in cells following crocidolite exposure.

Finally, the release of IL-1ß, IL-6 and TNF-α, pro-inflammatory cytokines, was determined by ELISA kits in control and treated A549. The results showed that the exposure to all experimental fibers ensued IL-1ß, IL-6 and TNF-α secretion (Table I), overall fluoro-edenite 27 and crocidolite. This latter promoted a more consistent release of these cytokines. On the contrary, tremolite induced less release compared to other fibers.

## Discussion

Many asbestos-like mineral fibers have been detected in the air of mountainous and volcanic areas of Italy and other parts of the world. These fibers have been suspected to be the cause of increased incidences of lung cancer and other lung diseases. However, the molecular mechanisms by which fibrous particulates induce cellular damage, as well the cellular

Table I. Pro-inflammatory cytokine concentrations measured by a specific sandwich enzyme-linked immunosorbent assay (ELISA) kit.

| | Control | Tremolite | Fluoro-edenite 19 | Fluoro-edenite 27 | Crocidolite |
|---|---|---|---|---|---|
| IL-1ß (pg/ml) | 61±3 | 48±2 | 102±4 | 237±7 | 623±10 |
| TNF-α (pg/ml) | 153±6 | 246±8 | 400±10 | 700±11 | 1569±22 |
| IL-6 (pg/ml) | 241±8 | 522±12 | 736±14 | 1886±25 | 2455±32 |

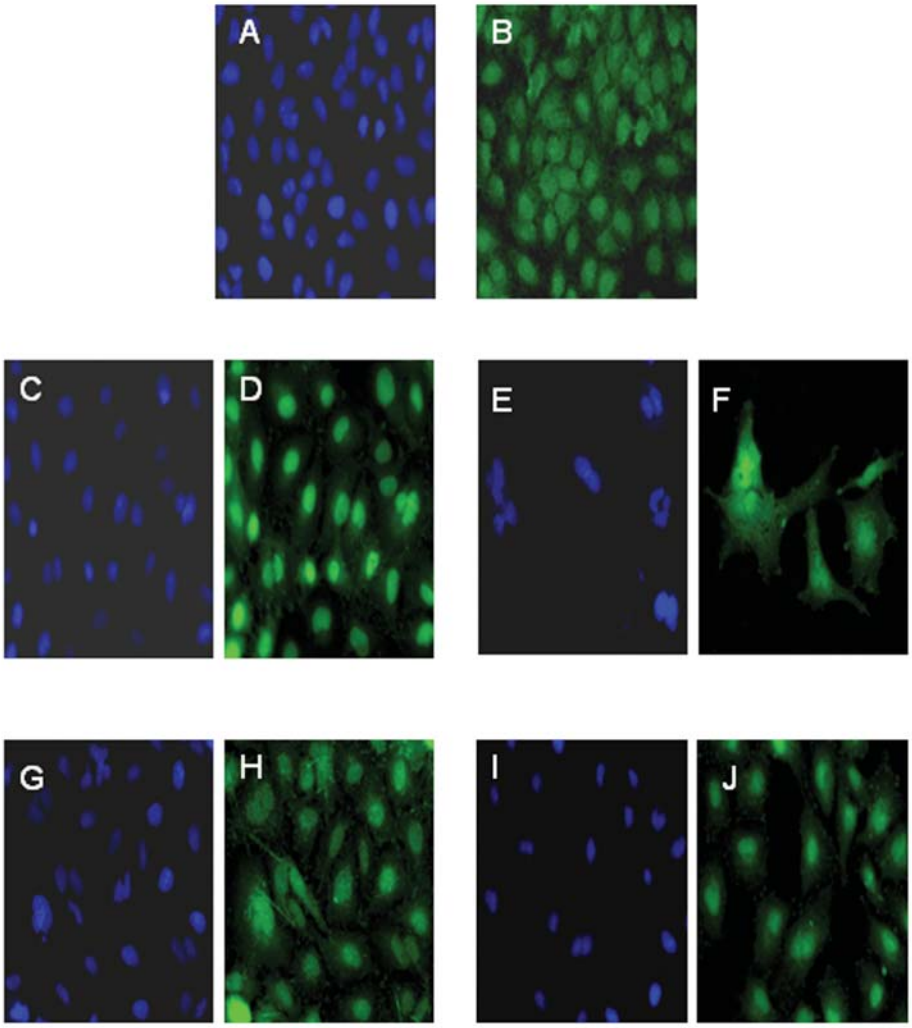


Figure 2. (A) PLC ß1 Control cells (DAPI). (B) PLC ß1 Control cells (FITC). (C) PLC ß1 Tremolite exposed cells (DAPI). (D) PLC ß1 Tremolite exposed cells (FITC). (E) PLC ß1 Fluoro-edenite 19 exposed cells (DAPI). (F) PLC ß1 Fluoro-edenite 19 exposed cells (FITC). (G) PLC ß1 Fluoro-edenite 27 exposed cells (DAPI). (H) PLC ß1 Fluoro-edenite 27 exposed cells (FITC). (I) PLC ß1 Crocidolite exposed cells (DAPI). (J) PLC ß1 Crocidolite exposed cells (FITC).

response to these particulates as it relates to carcinogenesis, remains unclear. Properties such as chemical composition, atomic structure, microtopography, surface charge, and dissolution and adsorption of ions and macromolecules have been associated with the biological effects of asbestos fibers. The surface activity of asbestos fibers may not only vary from one asbestos type to another, but also may change at different points on the same type of asbestos fiber. These variations may lead to the production of different levels and perhaps various species of radicals by individual asbestos types (17-19). Since asbestos fibers contain ferrous/ferric ions, it has proposed that the cytotoxic and genotoxic effects of asbestos fibers may be related to the generation of reactive oxygen species and/or reactive nitrogen species mediated by iron (20-22). Iron present in asbestos fibers is thought to be an important factor in the generation of $^{\bullet}OH$ produced from the reduction of $O_2$ or from the decomposition of $H_2O_2$. The signal transduction pathway for this phenomenon also involves PLC and PKC pathways (7). The various PLC isoenzymes appear to be activated by different receptors and distinct mechanism that initiate the cascade of molecular events leading to cellular responses (23). PLC ß1, which comprise the isoform α preferentially expressed in the cytosol, and isoform ß predominantly localized in the nuclei, is a G-protein-dependent phosphodiesterase at the plasma membrane and is activated and deactivated by phosphorylation in the nucleus (24). PLC γ1, widely distributed in bronchiolar epithelium, type I and II pneumocytes and fibroblasts of the interstitial tissue, is rapidly activated in response to growth factor stimulation and plays an important role in regulating cell proliferation and differentiation and may have a protective function during cellular response to oxidative stress.

To our knowledge this is the first study comparing the effects of fluoro-edenite 19 and 27 with those of tremolite and crocidolite by evaluating PLC ß1 and PLC γ1 expression in the A549 cells. Further comparisons have been investigated on the bases of the total iron and ferrous/ferric iron amount present in each fiber. Our data show an increased expression of both PLC ß1 and PLC γ1 in A549 cells treated with four asbestos-like fibers. PLC ß1 and PLC γ1 expression is not related to the total amount of the iron present in each

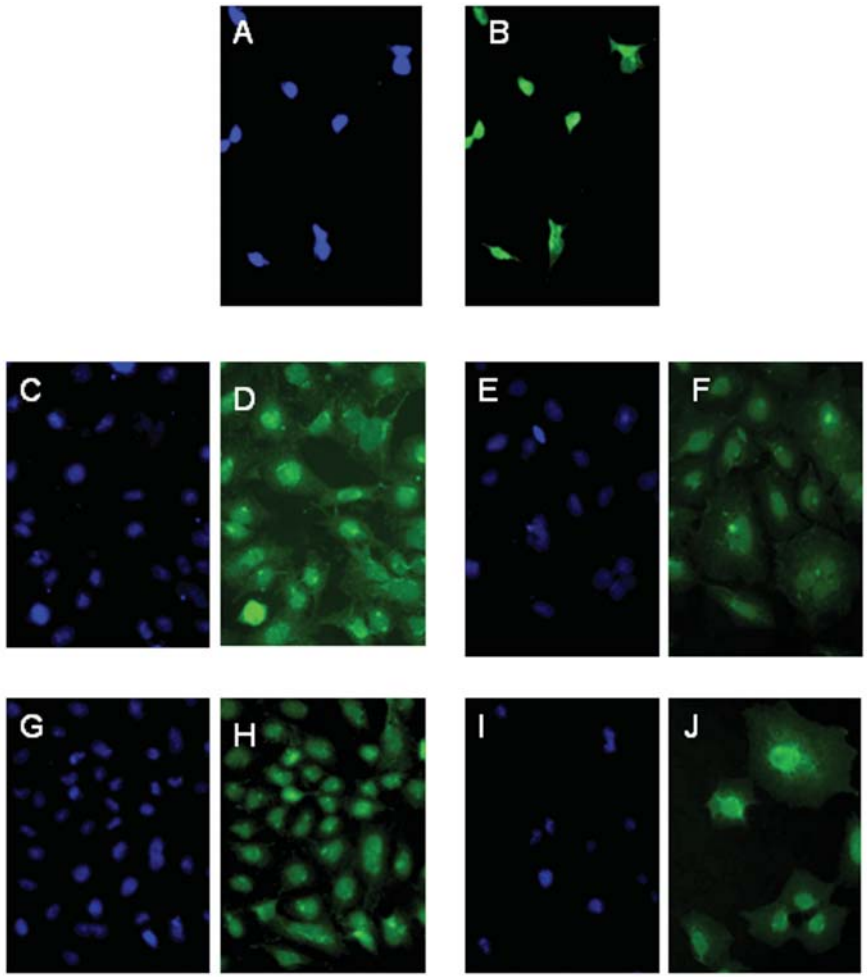


Figure 3. (A) PLC γ1 Control cell (DAPI). (B) PLC γ1 Control cells (FITC). (C) PLC γ1 Tremolite exposed cells (DAPI). (D) PLC γ1 Tremolite exposed cells (FITC). (E) PLC γ1 Fluoro-edenite 19 exposed cells (DAPI). (F) PLC γ 1 Fluoro-edenite 19 exposed cells (FITC). (G) PLC γ1 Fluoro-edenite 27 exposed cells (DAPI). (H) PLC γ1 Fluoro-edenite 27 exposed cells (FITC). (I) PLC γ1 Crocidolite exposed cells (DAPI). (J) PLC γ1 Crocidolite exposed cells (FITC).

fiber. Interestingly, increased PLCß1 expression levels are associated with highest amount of ferrous iron contained in each fiber. In contrast, this association was not observed by evaluating the PLC γ1 expression levels in cells treated with those fibers. The increased PLC expression levels indicate that PLC pathways may be involved during potential cellular transformation caused by asbestos-like fiber exposure. A translocation PLC γ1 from the cytoplasm to the nuclear compartment was also observed in the A549 cells after treatment. It has been shown that this trans-location is a marker of cell transformation or differentiation (1). In addition, after fiber treatment, in the present study we detected several multinucleated cells as a further indicator of cell transformation supporting the notion that asbestos-like fiber exposure may represent a high risk of cancer development.

Substantial data demonstrated that cytokines are produced by epithelial cells in response to inflammatory stimuli and cellular damage (11). It is also known that several cytokines are implicated in the pathogenesis of many diseases, including acute respiratory distress syndrome, pulmonary fibrosis, asthma, and cancer. Accordingly, the results of the present study show that IL-1ß, IL-6, and TNF-α levels are higher in the epithelial lung cells when treated with asbestos-like fibers in comparison to untreated cells. Cytokine increase was positive correlated to the amount of iron contained in each fiber. However, these data imply that no differences in cytokine release levels are observed by evaluating both ferrous and ferric iron contained in the analyzed fibers. In fact, A549 cells treated with tremolite that, among fibers herein studied, containing the lowest iron amount and the highest percentage of ferrous iron, show the lowest release of cytokines. While, crocidolite and fluoro-edenite 27 are able to promote the most secretion of IL-1ß, IL-6, and TNF-α. Several studies demonstrated that IL-1ß and TNF-α are potent mitogens to stimulate cell proliferation in various cell types and appear to exert their mitogenic effects through the activation of mitogen-activated protein kinase (MEK/MAPK) pathways to enhance the DNA synthesis (13). These stimulatory effects of cytokines are regulated by PKC, $Ca^{2+}$, PLC, and tyrosine kinase (13). However, our data suggest that cytokine release and PLC expression are not strictly linked.

Overall, the results of the present study support the notion that in the epithelial lung cells both PLC and cytokines may play an important role in the biological response to fluoro-edenite and the other asbestos fibers throughout different pathways.

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