

MICROTUBULE DISASSEMBLY INDUCED BY SENSITIZING HALOGENATED NITROBENZENE DERIVATIVES

M. F. LEUNG*, K. GEOGHEGAN-BAREK, G. B. ZAMANSKY and I. N. CHOU†

Department of Microbiology, Boston University School of Medicine, Boston, MA 02118, USA

Abstract—The potency of certain halogenated nitrobenzene derivatives to induce allergic contact dermatitis has been demonstrated in guinea-pigs and in man. As the first step towards understanding the mechanisms of cellular injury induced by these sensitizers, we have studied by fluorescence microscopy the effects of 13 halogenated (nitro)benzene derivatives on microtubule organization in mouse 3T3 fibroblasts and human AG1522 skin fibroblasts. Untreated cells have numerous microtubules distributed in a network fashion throughout the cytoplasm and extending to the cell periphery. Exposing cells for 3 hr to micromolar doses of halogenated nitrobenzene derivatives, which induce allergic contact dermatitis in guinea-pigs and man, resulted in a dose-dependent disassembly of microtubules. In contrast, incubation with 10–70 times higher doses of seven halogenated (nitro)benzene derivatives that do not sensitize guinea-pigs or man, had no discernible effect on microtubule organization of both cell types under identical assay conditions. Thus, for the 13 halogenated (nitro)benzene derivatives tested in this study, a 100% positive correlation exists between the sensitizing capacity as determined by *in vivo* tests on guinea-pigs and man (Landsteiner and Jacobs, 1936) and the ability to cause microtubule disassembly in cultured cells. These results may provide the basis for developing an *in vitro* screening assay for identifying other potential halogenated nitrobenzene sensitizers. In addition, these studies represent a new approach to investigating the mechanisms of contact sensitivity induced by simple chemicals.

Introduction

Certain halogenated nitrobenzenes are known to be potent sensitizers or irritants. Allergic contact dermatitis (ACD), a form of delayed hypersensitivity, is a common skin disease induced either by naturally occurring or by synthetic chemical sensitizers (Dupuis and Benezra, 1982). For example, 1-chloro-2,4-dinitrobenzene (CDNB), known to induce occupation-related ACD (Adams *et al.*, 1971; Malten, 1974; Zimmerman, 1970), is one of the most potent skin sensitizers and has been used as a model chemical to study experimentally induced ACD (Dupuis and Benezra, 1982).

Simple chemicals (haptens), like CDNB, penetrate the cell membrane and the skin layers (Carr *et al.*, 1984) and conjugate with cellular proteins (carriers) to form hapten–protein complexes (Baer and Rosenthal, 1978), which in turn trigger a series of immunological responses (Dupuis and Benezra, 1982; Katz, 1980; Polak, 1982). Although certain simple chemical sensitizers have been shown to form hapten–protein conjugates that are important in eliciting delayed hypersensitivity reactions (Hall and Smith, 1971; McFarlin and Balfour, 1973), the exact nature of cellular carrier protein(s) involved in ACD reactions has not been firmly defined. The cytoskeleton

collectively refers to a complex network of cytoplasmic filamentous structures consisting of three major components: 7-nm microfilaments, 10-nm intermediate filaments and 25-nm microtubules (MT). Microtubules are composed primarily of highly conserved, heterodimeric α and β subunits of tubulin (Mr 110 kDa) with minor amounts of microtubule-associated proteins. In addition to actin, which is the principal protein component of microfilaments, there are also a number of proteins that interact with or bind to actin. Several types of proteins belonging to intermediate filaments have been identified in different types of cells and vary depending on the tissue of origin. Keratins, vimentin, desmin, neurofilament protein and glial fibrillary acidic protein are the major protein species of intermediate filaments characteristic of epithelial cells, mesenchymal cells (fibroblasts), muscle, neuronal and glial cells, respectively (Alberts *et al.*, 1983; Bershadsky and Vasiliev, 1988; Clarkson *et al.*, 1984). The intricate distribution of cytoskeletal elements throughout the cytoplasm suggests that these cytoskeletal proteins may be good candidates for hapten/carrier conjugation.

The identification of sensitizing compounds depends mainly on the use of a limited number of animal tests (Marzulli and Maibach, 1987). For example, the sensitizing capacity of several halogenated nitrobenzenes, including CDNB, has been tested in guinea-pigs (Landsteiner and Jacobs, 1936), and the results have been reproduced in human subjects (Sulzberger and Baer, 1938). To evaluate whether the sensitizing capacity of halogenated (nitro)benzene derivatives correlates with the ability of these chemicals to induce cytoskeletal perturbations, we have undertaken a systematic study using

*Present address: Comprehensive Cancer Center, Yale University School of Medicine, New Haven, CT 06510, USA.

†To whom correspondence should be addressed.

Abbreviations: ACD = allergic contact dermatitis; CDNB = 1-chloro-2,4-dinitrobenzene; DMEM = Dulbecco's modified Eagle's medium; FBS = foetal bovine serum; MT = microtubule(s); PBS = phosphate buffered saline.

fluorescence microscopy to examine the effects of 13 halogenated (nitro)benzene derivatives, which have been extensively tested in both guinea-pigs and man (Landsteiner and Jacobs, 1936; Sulzberger and Baer, 1938), and shown a 100% positive correlation between sensitizing capacity and MT disruption in mouse 3T3 cells and human foreskin fibroblasts.

Materials and Methods

Cell cultures. Swiss 3T3 murine fibroblasts, a cell line originally established by Todaro and Green (1963), were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS, from Sigma Chemical Co., St Louis, MO, USA) and an antibiotic mixture of penicillin (250 U/ml) and streptomycin sulphate (250 µg/ml) in a humidified atmosphere of 7% CO₂. Quiescent cultures were obtained by plating $1.4\text{--}2.1 \times 10^3$ cells/cm² in DMEM containing 10% FBS and incubating for 24 hr as described by Schroder *et al.* (1980). The medium was replaced with DMEM containing 0.3% FBS for 48 hr followed by fresh DMEM containing 0.3% FBS. Cells were used in experiments 48–72 hr later.

Normal diploid human foreskin fibroblasts (strain AG1522), obtained from the Coriel Institute for Medical Research (Camden, NJ, USA), were grown in Eagle's minimal essential medium supplemented with 10% FBS, D-glucose (0.9 g/l), sodium pyruvate (0.66 mg/l), penicillin (110 U/ml) and streptomycin sulphate (110 µg/ml) as described by Zamansky (1986). Cultures were incubated at 37°C in a humidified atmosphere of 95% air:5% CO₂.

Fluorescence microscopic studies of the cytoskeleton. To examine the cytoskeletal organization by fluorescence microscopy, cells were plated onto glass coverslips (No. 1 thickness, 12 × 12 mm, Bradford Scientific, Inc., Epping, NH, USA) placed in culture dishes. Before use, coverslips were rinsed 10 times with deionized water purified through the Milli-Q Reagent Grade Water Systems (Millipore Corp., Bedford, MA, USA) and three times with 95% ethanol, and then stored in 95% ethanol. The coverslips were washed by dipping them into DMEM to remove residual ethanol and then placed in tissue culture dishes shortly before plating cells. The fluorescent labelling procedures used in this laboratory for visualization of MT have been described in detail (Li *et al.*, 1987; Zhao *et al.*, 1987) and are modified from those described by Solomon *et al.* (1979). Briefly, after removal of the medium, control and chemical-treated cells on glass coverslips were rinsed once with phosphate buffered saline (PBS) and incubated in an MT stabilizing buffer PM2G, pH 6.9 (100 mM-PIPES, 2 mM-EGTA, 1 mM-MgSO₄, 2 M-glycerol) for 1 min. The cells were fixed for 30 min in 3.7% formaldehyde (Aldrich Chemical Co., Inc., Milwaukee, WI, USA) freshly diluted in PM2G, and then incubated in PBS for 5 min followed by incubation with 0.1 M-glycine in PBS, pH 7.4, for 5 min to quench residual formaldehyde. The cells were extracted for 10 min with 0.3% NP40 freshly prepared in PBS and washed 3 times with PBS. All steps were performed at room temperature and all reagents were

warmed to room temperature before use. The extracted cells were stored at 4°C in PBS if they were not stained immediately for MT.

For MT visualization by indirect immunofluorescence, we used a polyclonal rabbit anti-tubulin antibody (Polysciences Inc., Warrington, PA, USA) as the primary antibody and rhodamine conjugated sheep anti-rabbit IgG (Organon Teknika-Cappel, West Chester, PA, USA) as the secondary antibody. The coverslips were drained, placed on a filter paper, overlaid with 20 µl of anti-tubulin antibody, and incubated at 37°C for 30 min in a moist chamber. The excess antibody was drained carefully using a paper towel and the coverslips were washed thoroughly by dipping 5 times in each of 5 beakers containing PBS. The coverslips were drained and overlaid with 20 µl of the secondary antibody, incubated in a moist chamber and washed with PBS as described above. The coverslips were washed once in deionized water before they were mounted on the slides using gelvatol (Monsanto, Indian Orchard, MA, USA) prepared as described by Jacobson *et al.* (1984). The slides were examined with a Nikon Optiphot microscope equipped with an epi-fluorescence attachment using the G-1A filter and Plan Apochromat 40 × oil objective. Photographs were taken using a UFX automatic exposure system (Nikon) with Kodak Tri-X pan film (ASA 400). The film was exposed at ASA 800 and 'push' developed to an effective ASA 1600.

Treatment of cells with chemicals. To monitor MT disruption induced by sensitizing or non-sensitizing analogue of CDNB, 3T3 cells or human foreskin fibroblasts were incubated in medium containing-micromolar concentrations of each chemical for 3 hr in the respective culture conditions as described above. The cells were processed and MT were visualized by indirect immunofluorescence as described. CDNB analogues (Table 1) that were examined include: 1-chloro-2,4-dinitrobenzene (CDNB), 1,3-dichloro-4,6-dinitrobenzene, 1,3-dichloro-5-nitrobenzene, 1,4-dichloro-2-nitrobenzene, 1,2-dichloro-4-nitrobenzene, *p*-chloro-nitrobenzene, *p*-dichlorobenzene, 1,2,4-trichlorobenzene, and 1,2,4,5-tetrachlorobenzene. These compounds were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). In addition, 1-bromo-2,4-dinitrobenzene, 1-iodo-2,4-dinitrobenzene, 1-fluoro-2,4-dinitrobenzene and 1-chloro-2,4,6-tri-nitrobenzene were also tested (obtained from ICN Biomedicals (Plainview, NY, USA), Fluka Chemical Corp. (Ronkonkoma, NY, USA) Sigma and Eastern Chemicals (Smithtown, NY, USA), respectively). All other compounds, unless otherwise stated, were obtained from Sigma.

Results

In preliminary experiments each chemical was tested at several doses on both types of cell to establish the optimal dose and time-response relationship. In the test illustrated (Plates 1 and 2) cells were exposed to µM doses of each compound for 3 hr and then processed for MT observation by fluorescence microscopy. Control 3T3 cells (Plate 1A) and human AG1522 foreskin fibroblasts (Plate 2A) contain numerous MT, which are organized in a network-like form extending from the microtubule organizing

Table 1. Correlation between microtubule disassembly in cultured cells and sensitizing capacity in guinea-pigs among various halogenated (nitro)benzene derivatives

Chemicals	Sensitization*	MT disassembly†
1-Chloro-2,4-dinitrobenzene	Positive	Positive
1-Bromo-2,4-dinitrobenzene	Positive	Positive
1-Iodo-2,4-dinitrobenzene	Positive	Positive
1-Fluoro-2,4-dinitrobenzene	Positive	Positive
1,3-Dichloro-4,6-dinitrobenzene	Positive	Positive
1-Chloro-2,4,6-trinitrobenzene	Positive	Positive
1,3-Dichloro-5-nitrobenzene	Negative	Negative
1,4-Dichloro-2-nitrobenzene	Negative	Negative
1,2-Dichloro-4-nitrobenzene	Negative	Negative
<i>p</i> -Chloronitrobenzene	Negative	Negative
<i>p</i> -Dichlorobenzene	Negative	Negative
1,2,4-Trichlorobenzene	Negative	Negative
1,2,4,5-Tetrachlorobenzene	Negative	Negative

*Sensitizing capacity of chemicals in guinea-pigs as described in Landsteiner and Jacobs (1936).

†Assays were carried out by exposing mouse 3T3 fibroblasts and human AG1522 fibroblasts for 3 hr to μM (as shown in Table 2) of chemicals that are 'positive' in causing allergic contact dermatitis in guinea-pigs and man. For 'negative' chemicals that do not sensitize guinea-pigs and man, each chemical was tested in standard MT disassembly assays at 100 μM for 3T3 cells and 40 μM for human AG1522 fibroblasts.

centre to the periphery of the cell. In general, two very different kinds of results were observed upon exposure of these cells to each of the 13 chemicals listed in Table 1. As shown in Plate 1 (B, C, D) and Plate 2 (B, C, D), all 6 'positive' halogenated nitrobenzene derivatives that are known to induce ACD (Landsteiner and Jacobs, 1936; Table 1), induced MT disassembly in a dose-dependent fashion in both cell types after 3 hr of exposure. For example, 2.5 μM -CDNB caused partial disassembly of MT whereas essentially complete MT disruption was observed in 3T3 cells treated with 3.0 μM -CDNB (compare Plate 1, A, B with C). For human skin fibroblasts, the CDNB doses that caused partial or complete disassembly of MT in 3 hr were 3.0 μM and 4.0 μM , respectively (Table 2). It should be noted that the effective dose range for each sensitizing derivative to induce MT injury is very narrow, and that the difference between the doses that induced partial or complete MT disassembly is $\leq 1 \mu\text{M}$ (Table 2). As shown in Table 2, only 1,3-dichloro-4,6-dinitrobenzene, the most potent MT disrupter among the 6 'positive' chemicals tested in our studies, was found

to induce MT disassembly which was accompanied by detachment of some cells (Plate 1D and Plate 2D). Whether the acute toxicity of this chemical may have contributed to the observed MT disassembly under the assay conditions used remains to be determined.

In sharp contrast, all seven 'negative' halogenated (nitro)benzene derivatives that do not induce ACD (Table 1), had no discernible effect on the organization of MT of both cell types, even in cells exposed to chemicals at doses as high as 100 μM for 3T3 cells (Plate 1, D, E, F) or 40 μM for human skin fibroblasts (Plate 2, D, E, F). It should be noted that these concentrations of non-sensitizers represent, respectively, 33–70 times and 10–30 times higher than the doses of sensitizers necessary to induce MT disassembly in 3T3 cells and human skin fibroblasts. Thus, the results from the *in vivo* sensitization tests agree completely with those from MT disassembly assays using two types of cultured cells.

Discussion

A 100% positive correlation between the sensitizing capacity of halogenated (nitro)benzenes and their ability to induce MT disassembly in a dose-dependent fashion was demonstrated (Tables 1 and 2). CDNB is one of the most potent skin sensitizers and has been used as a model compound to study mechanisms of experimentally induced ACD (Dupuis and Benezra, 1982). The sensitizing capacity of halogenated (nitro)benzene derivatives (Table 1) has been demonstrated in guinea-pigs and in man.

It has been shown that small sensitizing chemicals and carrier proteins form hapten-protein conjugates that are important in inducing and eliciting the delayed hypersensitivity reactions associated with allergic contact dermatitis (Hall and Smith, 1971; McFarlin and Balfour, 1973). However, the actual cellular carrier proteins remain to be identified. Chemical penetration studies indicate that simple chemicals like CDNB can penetrate through the dermis layer within 6 hr of application onto the human skin (Carr *et al.*, 1984). The complex network of cellular MT, which are distributed intricately throughout the cytoplasm in all eukaryotic cells, is

Table 2. Determination of the dosage of sensitizing chemicals required to induce MT disassembly in different types of cultured cells

Chemicals*	MT disassembling dosage (μM)†	
	3T3 cells	Human fibroblasts
CDNB	3 (2.5)	4 (3)
BDNB	2.5 (2)	3 (2.5)
IDNB	2.25 (2)	3 (2)
FDNB	2 (1.5)	4 (3)
1,3-DC-4,6-DNB	1.3 (ND)	1.3 (ND)
1-C-2,4,6-TNB	3 (2.75)	3.5 (3)

ND = not determined

*CDNB, 1-chloro-2,4-dinitrobenzene, BDNB, 1-bromo-2,4-dinitrobenzene; IDNB, 1-iodo-2,4-dinitrobenzene, FDNB, 1-fluoro-2,4-dinitrobenzene; 1,3-DC-4,6-DNB, 1,3-dichloro-4,6-dinitrobenzene; 1-C-2,4,6-TNB, 1-chloro-2,4,6-trinitrobenzene.

†Mouse 3T3 cells and human AG1522 fibroblasts were exposed to various doses of each chemical for 3 hr and then processed for visualization of MT by immunofluorescence. The minimal dose of each chemical required to cause complete MT disassembly was determined for each cell type. Doses of each chemical that induced partial MT disassembly are shown in parentheses.

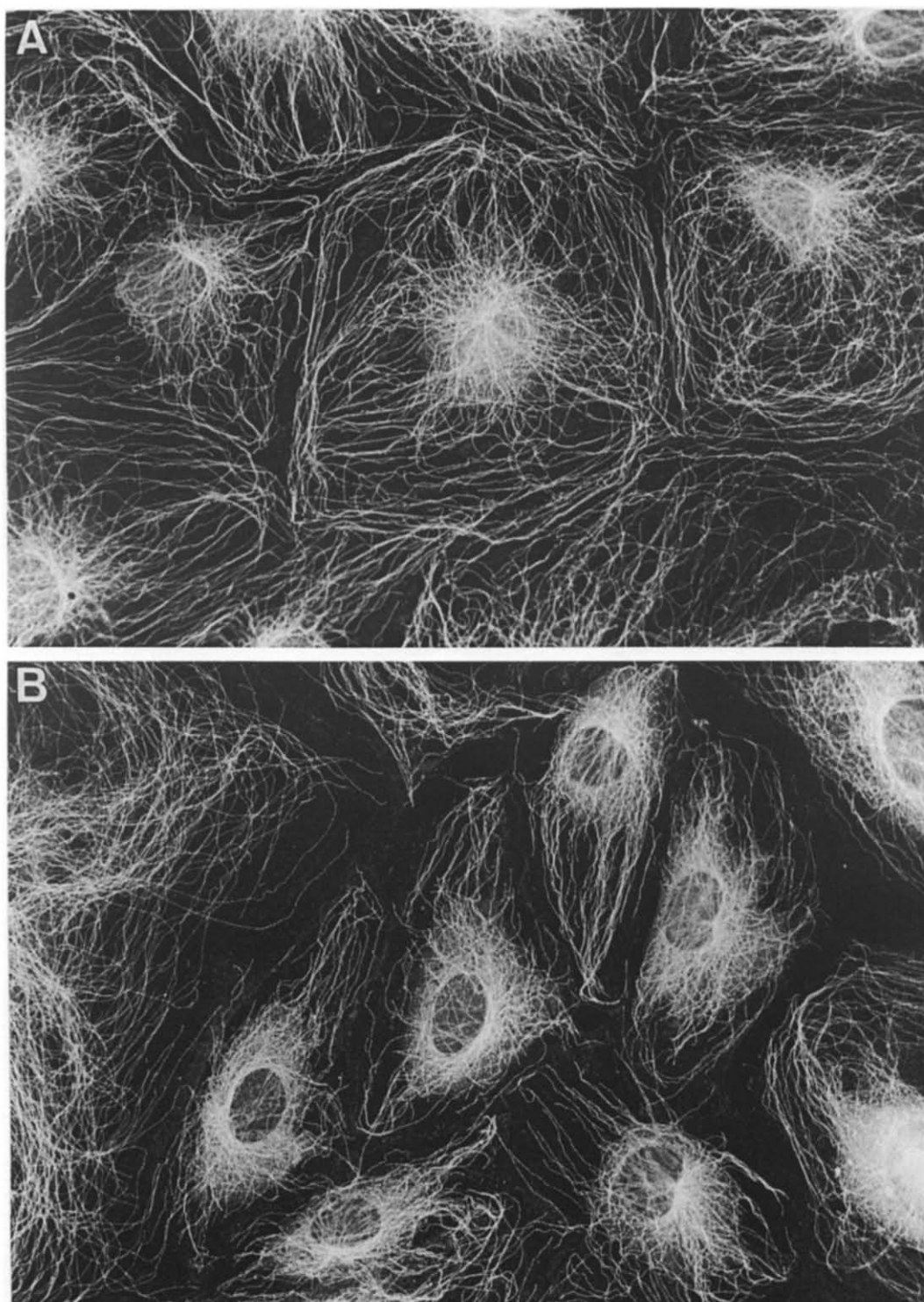


Plate 1 (A) and (B)

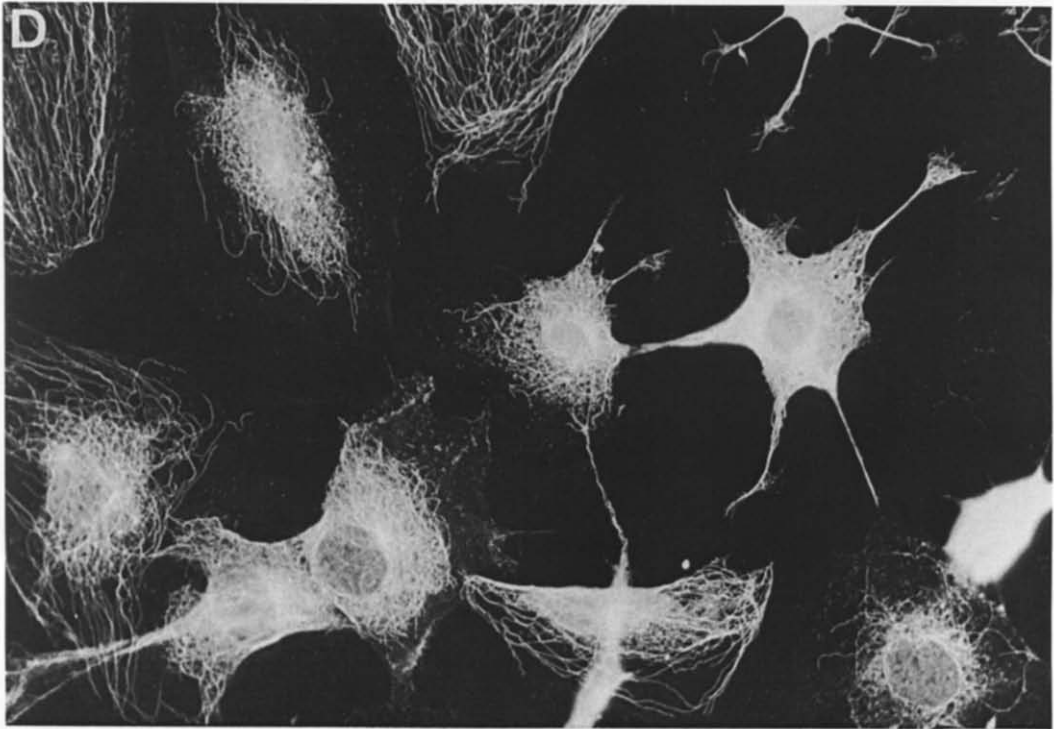
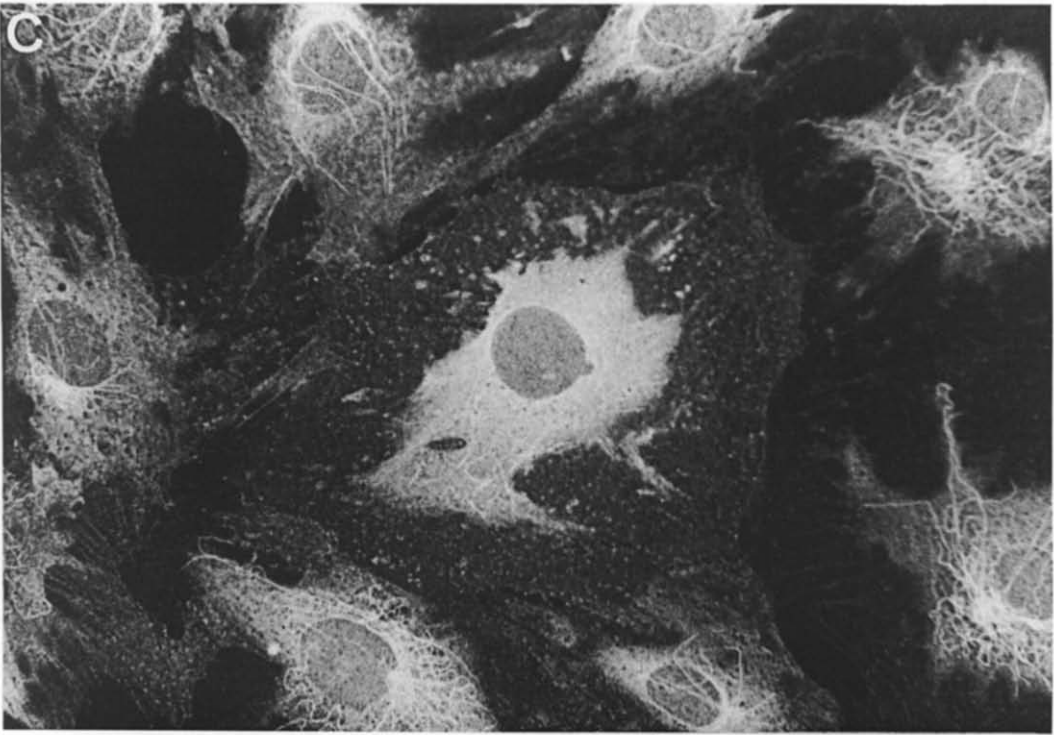


Plate 1 (C) and (D)

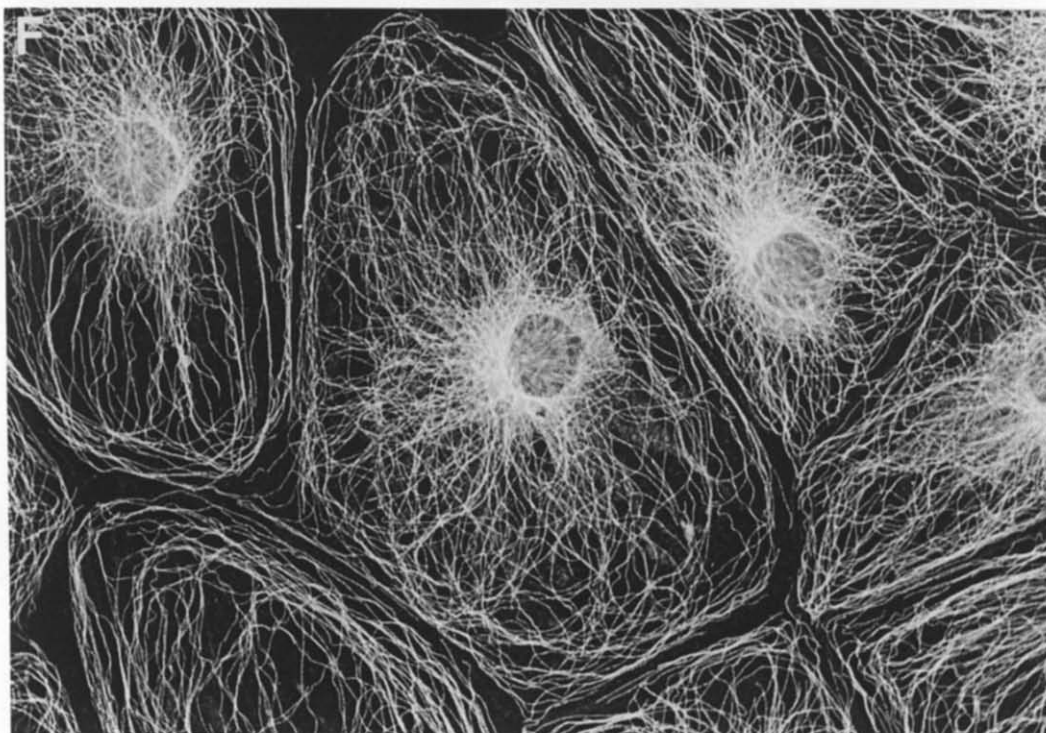
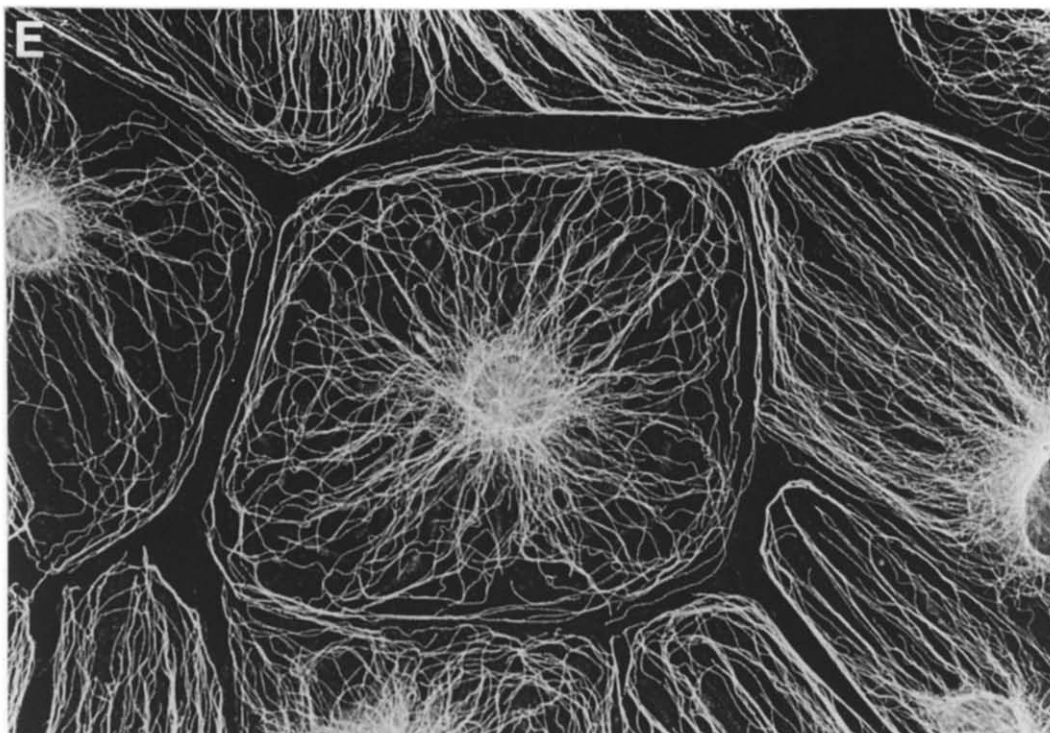


Plate 1 (E) and (F)

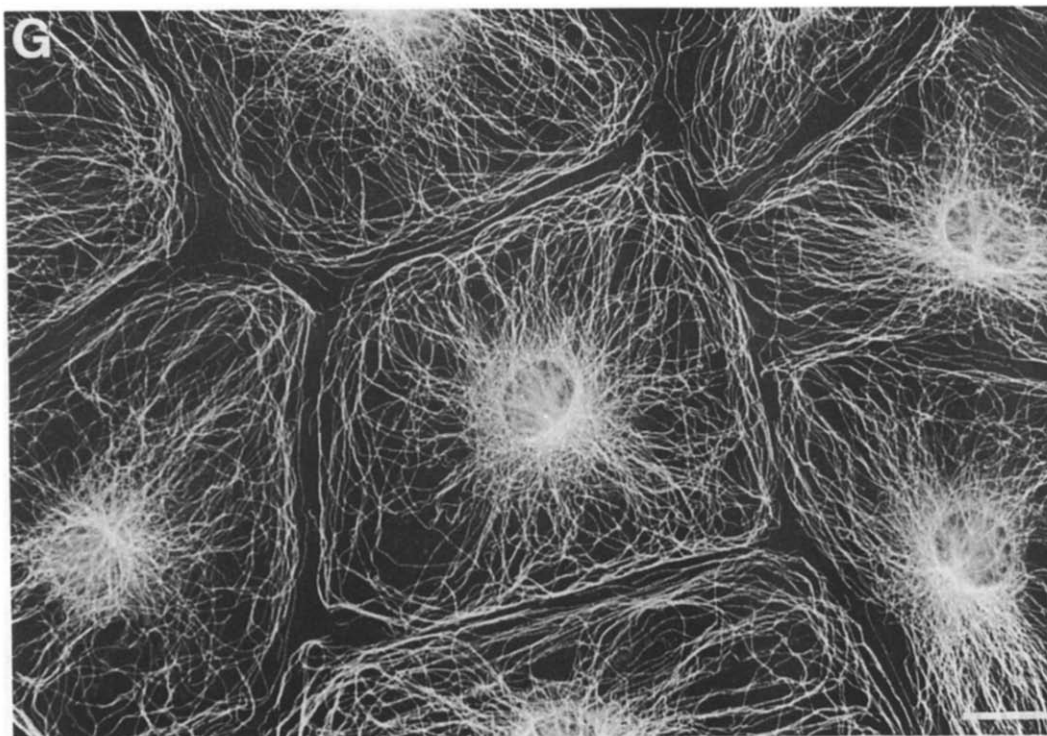


Plate 1 (G)

Plate 1. Effects of halogenated (nitro)benzene derivatives on MT organization of 3T3 cells. Untreated cells (A), and cells exposed for 3 hr to 2.5 μM -CDNB (B), 3 μM (C), 1.3 μM -1,3-dichloro-4,6-dinitrobenzene (D), 100 μM -1,2-dichloro-4-nitrobenzene (E), 100 μM -*p*-chloronitrobenzene (F) and 100 μM 1,2,4,5-tetrachlorobenzene (G) were processed for MT visualization using immunofluorescent labelling procedures. Note that the last three compounds that are unable to cause MT disassembly (E, F, G) are also 'negative' in the skin sensitization assay (see Table 1). For 'positive' sensitizers, partial MT disassembly was observed in (B) whereas severe MT disruption was evident in both (C) and (D).

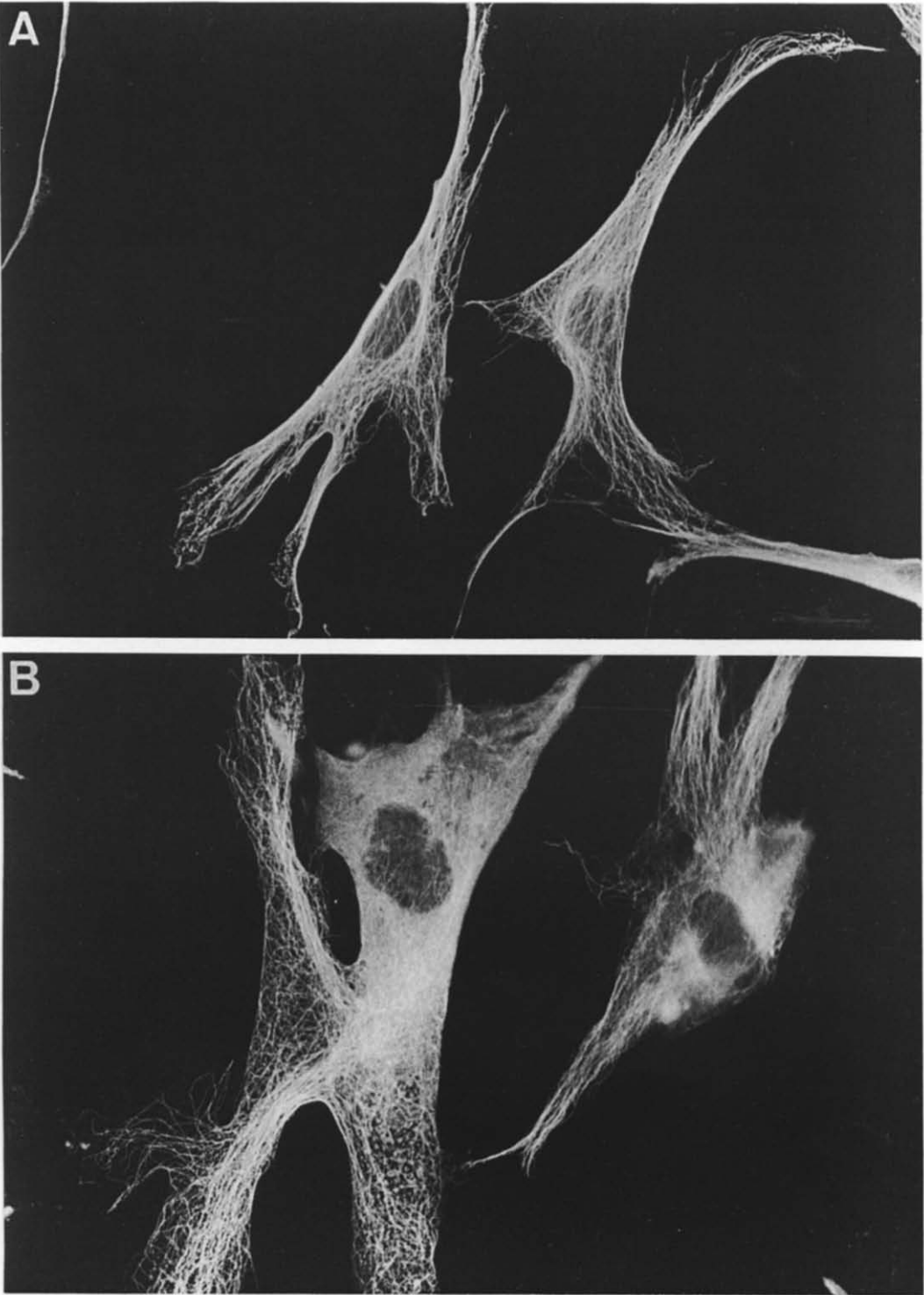


Plate 2 (A) and (B)

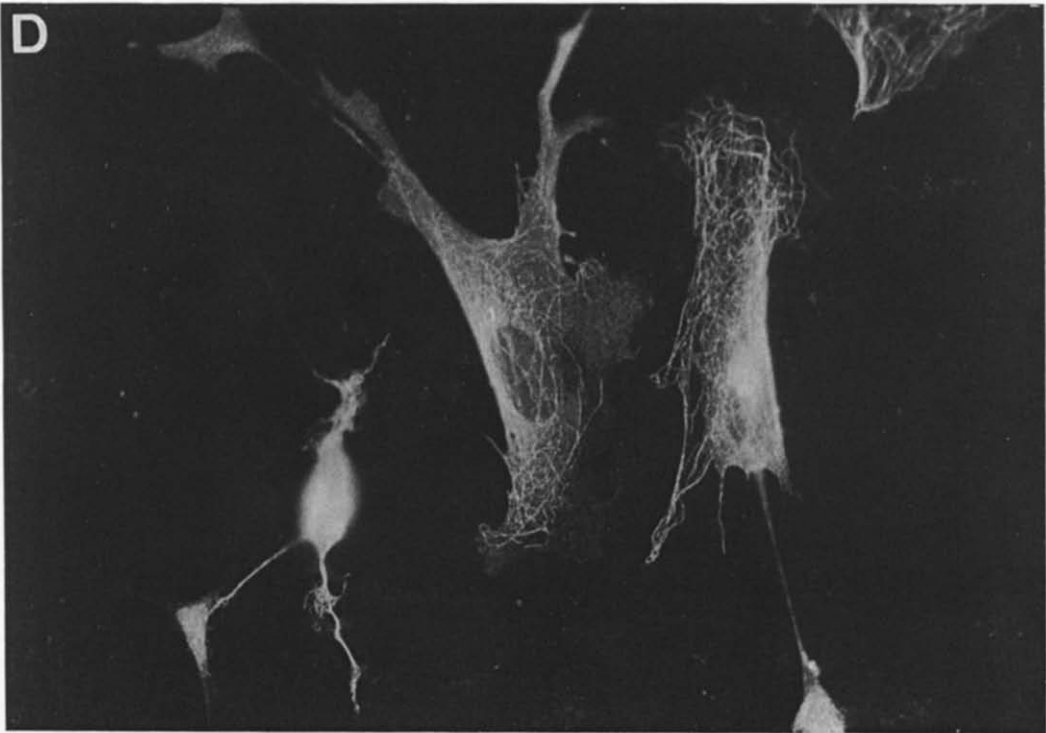
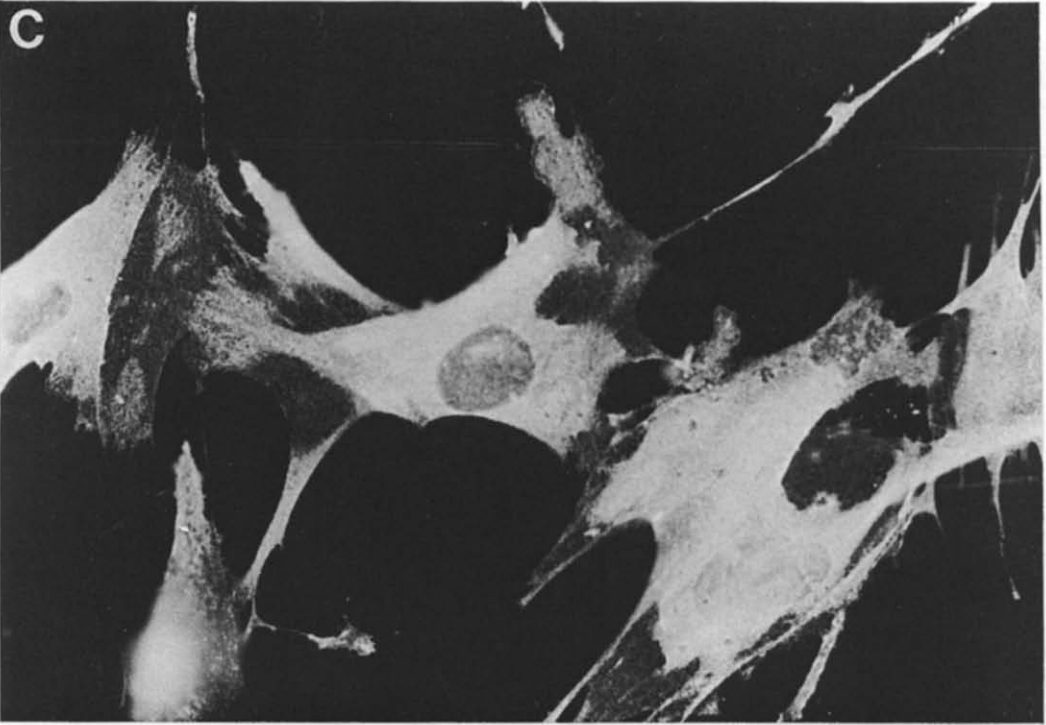


Plate 2 (C) and (D)

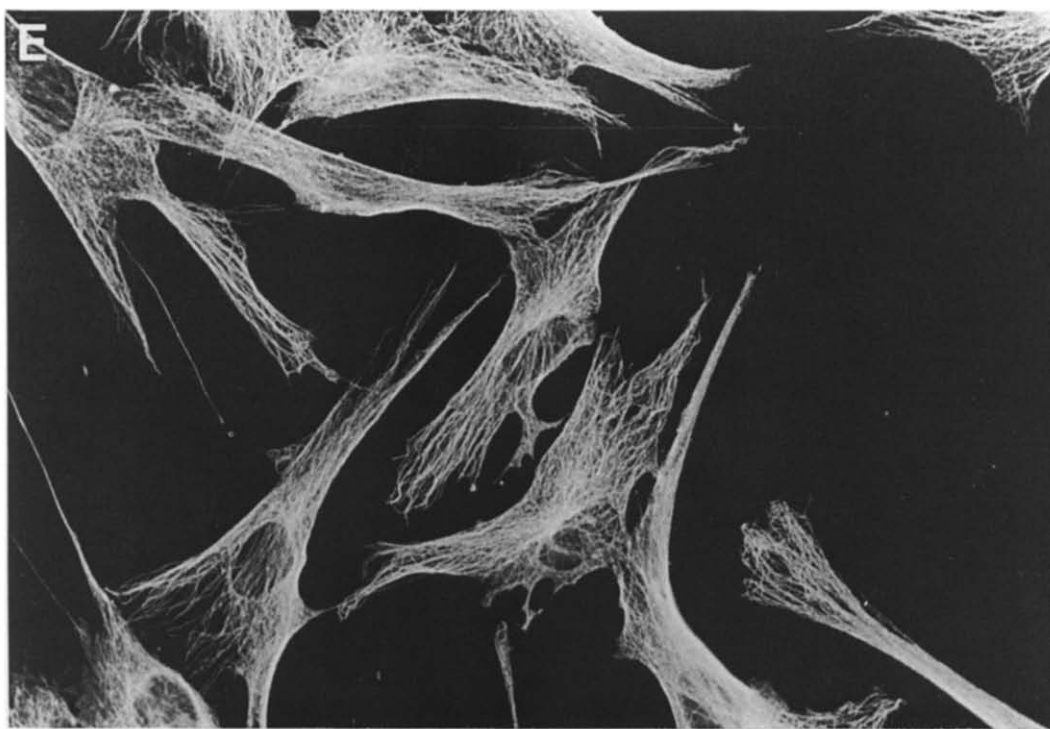


Plate 2 (E) and (F)



Plate 2 (G)

Plate 2. Effects of halogenated (nitro)benzene derivatives on MT organization of human AG1522 foreskin fibroblasts. Untreated cells (A), and cells exposed for 3 hr to 3 μM -CDNB (B), 4 μM (C), 1.3 μM -1,3-dichloro-4,6-dinitrobenzene (D), 40 μM -1,2-dichloro-4-nitrobenzene (E), 40 μM -*p*-chloronitrobenzene (F) and 40 μM -1,2,4,5-tetrachlorobenzene (G) were processed for MT visualization using immunofluorescent labelling procedures. Note partial (B) and complete (C) MT disruption in cells exposed to CDNB at 3 μM and 4 μM , respectively. Whereas MT organization was essentially unaffected in cells exposed to 'negative' sensitizing compounds (E, F and G), severe MT disruption was clearly evident in cells treated with another 'positive' sensitizer (D).

probably highly accessible to skin sensitizers once they enter the cells. As summarized in Table 1, these studies demonstrate a perfect correlation between the sensitizing capacity of 13 halogenated nitrobenzene derivatives tested *in vivo* (guinea-pigs and man) and the ability of these chemicals to induce MT disassembly observed *in vitro* (cultured cells). This positive correlation suggests that MT proteins may be an important carrier(s) capable of forming conjugates with this family of skin sensitizers. Furthermore, these results also suggest that an *in vitro* assay based on the ability to induce MT disassembly in cultured cells may be developed for screening other potential sensitizing agents. In addition, this study represents a new approach to studying the molecular mechanisms of ACD induced by simple sensitizing chemicals.

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