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Ultrastructural changes induced by α-sarcin in a human pulmonary tumor grown in naked mice

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Summary. α-Sarcin is a cytotoxic polypeptide produced by *Aspergillus giganteus*. It suppresses protein synthesis in yeast and wheat germ extracts and has a purine-specific RNase activity. The substance has been tested for its antitumor properties in a series of induced tumor systems in mice such as sarcoma and carcinoma among others.

Although some of the in vitro effects of α-Sarcin on certain cellular components have been elucidated, the biological effects leading to cellular damage are still obscure.

In this work we analysed the morphological changes in tumor cells derived from human pulmonary adenocarcinoma heterotransplanted and grown in naked mice, induced shortly (24 hours) after a single intratumoral injection of α-Sarcin (0.4 mg/tumor). The results obtained were: 1) swelling of mitochondria; 2) cell necrosis with partial removal of necrotic cells by phagocytosis; 3) thickening of interlobular connective tissue; 4) hyperplasia of globet-cell-like clear cells. The mode of action concerning these cellular changes is presently uncertain.

In view of the severity of these structural alterations it seems conceivable that α-Sarcin may enter the cell undergoing interactions with different intracellular structures. This would require a selective membrane permeabilization, perhaps induced upon formation of complexes with negatively-charged membrane phospholipids.

Key words: α-Sarcin, Human pulmonary tumor, Naked mice, Ultrastructure

Introduction

The cytotoxin α-Sarcin (Olson, 1963) is a small protein consisting of a single polypeptide chain extracted from the mould *Aspergillus giganteus*. It exhibits antitumor activity against different types of tumors within doses that are untoxic. Treatment of mice bearing sarcoma 180 with α-Sarcin have shown a sixfold difference between the minimal inhibitory level and the toxic level (Olson and Goerner, 1965; Olson et al., 1965). The amino acid sequence of α-Sarcin, which has an Mr of 16,987 (Sacco et al., 1983), is similar in limited regions to purine-specific RNase isolated from other fungi, and may harbour structural elements, which are closely related to the structure of the RNase T1 from *Aspergillus oryzae* (Erdmann et al., 1989; Ulbrich et al., 1989). The α-Sarcin protein sequence is very homologous (86%) to the *Aspergillus restrictus* toxins mitogillin and restrictocin (López-Otin et al., 1984).

It has been shown that α-Sarcin suppresses protein synthesis in yeast and wheat germ extracts and that it makes a specific cut in 28S rRNA of isolated yeast ribosomes (Schindler and Davies, 1977). The ribosome-inactivating effects of α-Sarcin, restrictocin, and mitogillin have been studied in great detail (Fernández-Puentes and Vázquez, 1977; Conde et al., 1978; Hobden and Cuntliffe, 1978; Fando et al., 1985; Ulbrich et al., 1988). The precise position of the 28S rRNA cleavage site on rat liver polysomes was determined (Endo and Wool, 1982) and it was shown that the toxin exhibited these very specific cleavage 393 nucleotides from the 3'-terminus of 28S rRNA yielding 3'-phosphate and 5'-hydroxyl termini. For unprotected RNA, after extensive digestion, cleavages after all the adenines and guanines have been reported for both single- and double-stranded regions (Endo et al., 1983).

Little is known about the cellular or biological effects of α-Sarcin. The effect of α-Sarcin on picornavirus-infected cells was investigated and it could be demonstrated that 90% inhibition of protein synthesis occurred at 1×10^{-7} M, whereas no inhibition of translation was observed in uninfected cells even at a concentration of 4×10^{-5} M. Similar results were obtained with HeLa cells infected by adenovirus type 5 and with BHK cells infected by Semliki Forest virus (Fernández-Puentes and Carrasco, 1980), suggesting that certain cytotropic or

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cytopathic viruses may be involved in the procedure of membrane permeabilization. Increased entry of macromolecules, such as α-Sarcin under certain permeabilization conditions due to changes in the concentration of the divalent cations $Mg^{2+}$and Ca^{2+} however, was not affected by nifedipine, dibucaine or mepacrine, but was partially inhibited by NH_4Cl, amantadine and chloroquine (Otero and Carrasco, 1987).

The specific effects of α-Sarcin on whole cells have not yet been examined. Therefore, in order to test α-Sarcin activity in a living cell, it was injected into *Xenopus* oocytes (Ackerman et al., 1988). In oocytes α-Sarcin does not behave like a general nuclease cleaving after all the purines, nor does it operate by any other means such as initiating proteolytic digestion of endogenous oocyte proteins. But instead it cleaves a single phosphodiester bond in the putative α-Sarcin recognition sequence (Wool, 1984) near the 3'end of 28S rRNA and causes a rapid decline in oocyte protein synthesis for soluble cytoplasmic proteins, similar in effect to injection of cycloheximide or puromycin.

In addition, the substance could have a number of non-specific cellular effects on certain cytoplasmic components other than ribosomes, thus causing different cellular changes.

Whether these effects of α-Sarcin are dependent on its passage across the cell membrane or could be carried out without entering the cytoplasm, is still unknown.

In the present study the effects of α-Sarcin on the cell morphology after intratumoral application of a single dose have been examined using light microscopic and electron microscopic techniques to characterize direct cellular alterations.

Materials and methods

Aspergillus giganteus was grown at 30°C for 96 hrs in a rotary shaker, at 200 rpm, in medium: 2% soluble starch, 0.75% meat extract, 1% bacto peptone and 0.5% NaCl, inocculated with approximately 6×10^7 spores/400 ml medium in 1000 ml flasks.

Fractions obtained either by a batch procedure or ion exchange chromatography on CMC 52, the crude extract, were resolved further by gel filtration and applied to a column (4 x 85 cm) of Ultragel AcA54, equilibrated with 50 mM NaH_2PO_4, pH 7.5. The flow rate was 65 ml/h and 15 ml/fractions were collected. Alternatively, the crude extract was separated by FLPC cation exchange chromatography on a Mono S column (Pharmacia). Bound protein was eluted with a linear gradient of 0.1 -0.4 M NaH_2PO_4, pH 6.0. Elution of the protein was monitored by determining the absorption at 280 nm. The molecular weight and the purity of the isolated protein were estimated by gel electrophoresis in sodium dodecyl sulphate (Laemmli, 1970). Prior to application, the protein was dissolved in a sterile 0.9% NaCl solution to a final concentration of 2 mg/ml.

The tumor was originally derived from a human pulmonary adenocarcinoma which was heterotransplanted to naked mice and grown progressively. Tumor-bearing mice (1st generation) were sacrificed with a CO_2 overdose, the tumors were dissected free of the capsule and minced into approximately 0.5 mm^3 fragments in Hank's balanced salt solution. The resulting coarse suspension was subcutaneously injected (0.5 ml/mouse) into healthy naked mice (8-10 weeks of age at the time of tumor transplantation). The animals given the injection developed tumors rapidly, exhibiting progressive growth.

Two weeks after tumor transplantation, the mice received α-Sarcin (0.2 ml/mouse) directly injected into the tumor of approximately 2 cm^3 in size giving an estimated intratumoral concentration of 10^{-5} M α-Sarcin. The control mice were treated in the same manner using 0.9% NaCl solution. In order to minimize traumatical cell lesions the injections were carried out quite slowly with a very thin needle.

24 hours later the mice were sacrificed, the tumors were removed and sectioned into two parts. One was preserved in Bouin's solution (1:20 picric acid/acetic acid) for light microscopy, whereas the other one was fixed in 2% glutaraldehyde, 0.5 g tannic acid and 0.1 M phosphate buffer followed by 1% OsO_4 buffered in 0.2 M phosphate buffer. Ultrathin sections were stained with uranyl acetate and lead citrate and examined on a Siemens Elmiskop 101 electron microscope.

Results

Macroscopically, the tumors were slightly nodular, whitish-grey and somewhat translucent with opaque areas. The treated tumors appeared smaller in size and not as firm as the untreated ones. Histological examination yielded several important findings. At the light microscopic level, the untreated tumors showed characteristic glandular structures with a central lumen that often contained opaque homogeneous material. The glands were lined by pseudostratified epithelium that mainly consisted of tall columnar cells and several goblet-cell-like clear cells which were scattered throughout the epithelial layer (Fig. 1a-c). The surrounding interlobular connective tissue seemed to be poorly developed. Numerous mitotic figures could be recognized in the epithelium, indicating rapid growth activity.

The treated tumors showed an increase in the number of the globet-cell-like clear cells which were distinguished by a fine vacuolated appearance. Abundant cell necrosis could be seen all over the epithelial layer (Fig. 1d-f). Some of the necrotic cells became engulfed by adjacent epithelial cells. There we could see all stages of cytolysis. In the final phase of phagocytosis the cellular membrane and the membranes of cell organelles were vastly fragmented. There was an extreme swelling of all membrane-bordered cell components and the nucleus showed marked pyknosis. In this case, the whole cell mass looked like a heap of cell debris, where the intracellular detail had been completely lost (Fig. 2a-b). The necrotic processes seemed to be the most striking feature of the

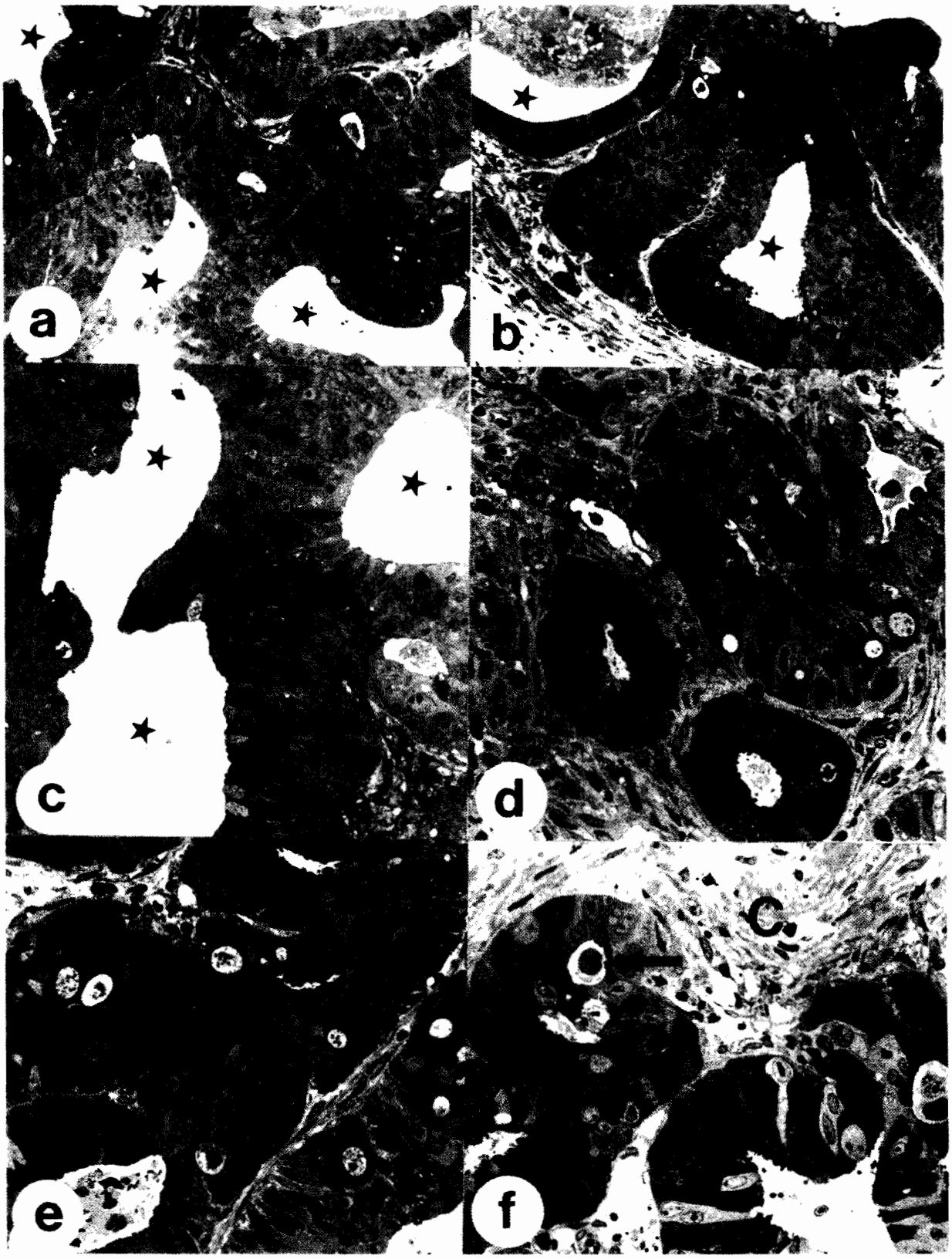

Fig. 1.a-c. Light microscopic picture of NaCl-treated tumor originating from bronchial mucous glands showing a glandular structure dominantly lined by columnar cells with some isolated globet-cell-like clear cells scattered between them, and scanty interlobular connective tissue. The star indicates the lumen of glandular structures. a and b: × 250, c: × 400.
d-f. Light microscopic picture of α-Sarcin-treated tumor demonstrating numerous necrotic cells (thick arrow) and enlargement of interstitium (C) which takes up a much greater area than the untreated tumor. Electron-dense cells are marked by a thin arrow. × 400

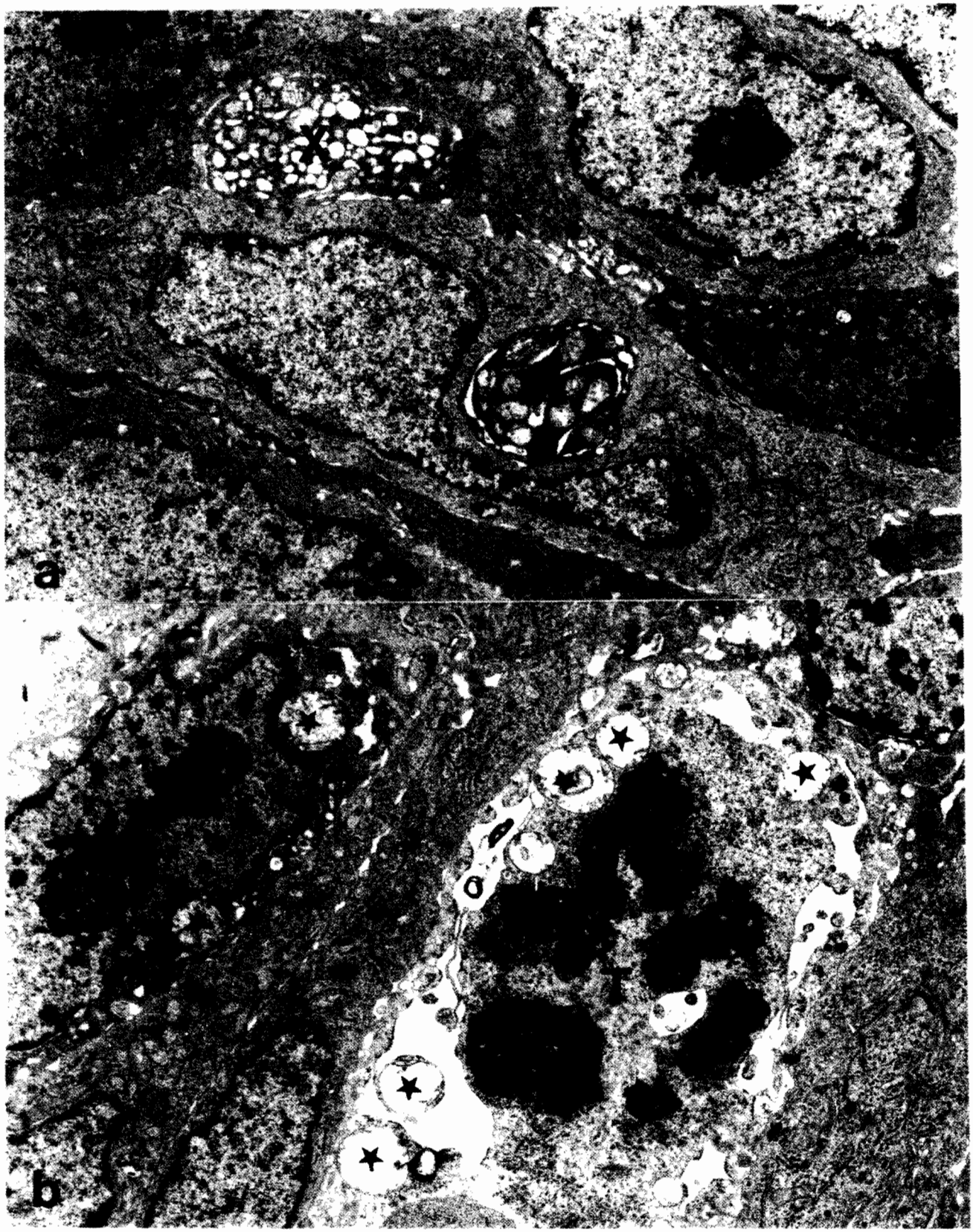

Fig. 2.a. Electron micrograph of an α-Sarcin-treated tumor showing a cell with swollen organelles and a phagocytised fragmented cell with similar alterations (X). × 9,000. **b.** Cells with swollen mitochondria (marked by a star). The cell on the right side of the picture shows an unusual telophase stage of mitosis (T). × 9,000

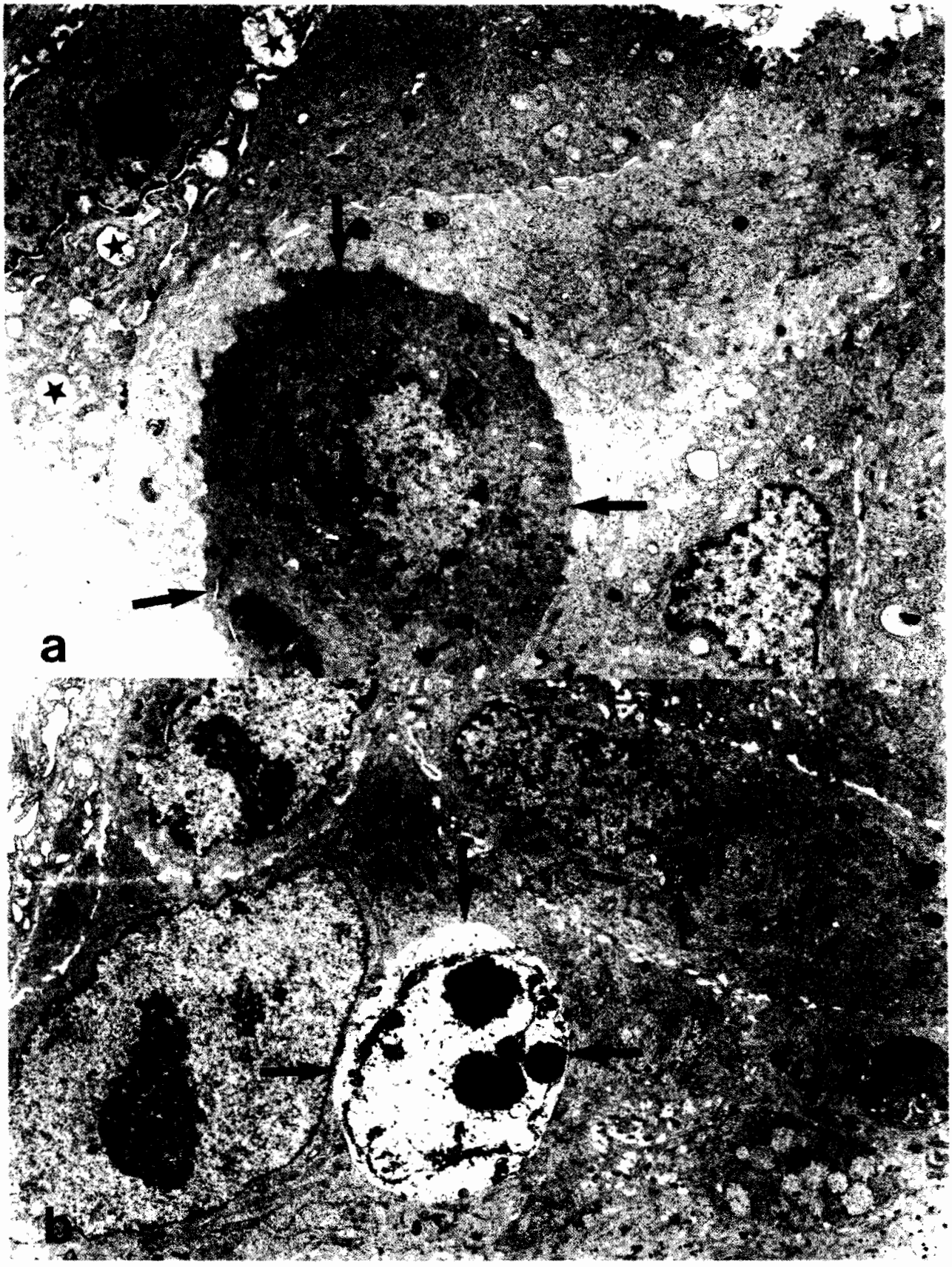

Fig. 3.a. Electron micrograph of an α-Sarcin treated tumor showing a dense cell at the top of the picture with numerous swollen mitochondria (marked by a star). An engulfed necrotizing cell within the cytoplasm of the phagocytic cell is marked by thick arrows. x 6,000. **b.** Engulfed cells at different stages of cytolysis. An electron-lucent cell during necrolysis within the cytoplasm of the phagocytic dense cell (marked by thick arrows). Two smaller dense cells, allready fragmented within the phagocytic cells are marked by an thin arrow. x 6,000

morphological changes induced by α-Sarcin. This caused an imbalance between epithelium and connective tissue. The areas with interstitial connective tissue were enlarged and the epithelial part was reduced. Numerous fibroblasts showing well-developed rough endoplasmic reticulum were observed.

Additional information concerning the intracellular changes was obtained from the electron micrographs. The most remarkable ultrastructural alterations were swellings of mitochondria in some cells, the cristae of which were disrupted, had partially disappeared and the matrix had become less densely packed. The mitochondria were occasionally filled with amorphous material (Fig. 3a-b). Not only these cells which were about to necrotize but many others displaying mitochondrial swellings and furthermore some morphologically intact cells were already engulfed by adjacent epithelial cells.

Discussion

Numerous investigations dealing with α-Sarcin mainly accentuate the ribosome-inactivating properties responsible for the inhibition of protein synthesis. Little attention has been paid to other possible cellular effects of this substance.

Many reports, including the one presented here, indicate that α-Sarcin is a cytotoxic substance with a broad spectrum and may cause several intracellular changes other than the inactivation of ribosomes. No evidence exists to date of a special membrane passage of α-Sarcin through which the substance could enter the cell, as some other ribosome-inactivating proteins of type 2 easily do (Montecucchi et al., 1989).

Hydrolysis of a specific phosphodiester bond in the 28S rRNA requires a direct intracellular interaction with the ribosome resulting in an efficient inhibition of translation as confirmed in cell-free systems but not in intact cells, unless they are permeabilized (Fernández-Puentes and Carrasco, 1980).

Recent studies using synthetic model membranes (Rietveld et al., 1983) and bacterial cytoplasmic membranes (Nesmeyanova, 1982; De Vrije et al., 1988) suggest that proteins can selectively penetrate cell membranes due to lipid-protein interaction resulting in charge alteration of phospholipids leading to promotion of transmembrane movement of both phospholipid and protein molecules.

The positively-charged α-Sarcin, exhibiting an isoelectric point larger than 10.65 (Erdman et al., 1989; Ulbrich et al., 1989) apparently behaves in a similar way and forms complexes with negatively charged phospholipids resulting in permeabilization of cell membranes (Gasset et al., 1989). Another possibility for altering the permeability could be an activation of membrane phospholipase C by α-Sarcin. The role this lipase plays in the control of permability has been well explored (Otero and Carrasco, 1988). Using exogenous phospholipase C, these authors have been able to demonstrate an effect on the permeability, suggesting that the enzyme is able to modify phospholipids resulting in the permeabilization of the membrane for protein and other macromolecules.

A hypothetical activation of cellular phospholipase C induced by α-Sarcin would reveal the same result. On the other had, this activation leads to Ca^{2+} release from intracellular sources causing an alteration of membrane permeability probably due to Ca^{2+}–lipid interaction (Yguerabide, 1980). This binding of Ca^{2+} to the anionic phospholipids would result in a neutralization of the negative charges (Yguerabide and Yguerabide, 1985) which in turn minimizes the electrical resistance of the membrane and renders it permeable to the proteins (Fernández-Puentes and Carrasco, 1980).

All these investigations were carried out whilst the membrane permeability had been previously enhanced by different kinds of agents such as viruses (Fernández-Puentes and Carrasco, 1980) and ionophores (Otero and Carrasco, 1987). Hence, the data obtained do not indicate the free passage of α-Sarcin through intact biological membranes under normal conditions *in vivo*. Nevertheless, as to how and whence, i.e., extra- or intracellularly, α-Sarcin may display its effect on different cell structures, the data presented in this communication clearly demonstrate two very serious intracellular alterations which perpetually lead to cell death. Some of our results, such as swelling of mitochondria and cell necrosis, could be interpreted easily according to the known cytotoxical properties of α-Sarcin, which may act similar to some well-known mitochondrial toxins as CCl_4 (Christie and Judah, 1954) and different snake and bee venoms (Habermann, 1954) causing an inhibition of oxidative phosphorylation and ATPase activity as well. The other findings, including the thickening of interlobular connective tissue and increase in the number of goblet-cell-like clear cells, remain unexplained.

It is quite likely that the enlargement of connective tissue could be due to the rapid diminution of epithelium induced by α-Sarcin. In view of the short exposure time (24 hours) of the tumor to this substance, a probable direct stimulation of the connective tissue by α-Sarcin can be of no consequence for this result.

Considering the role connective tissue plays in the process of tumor defense this finding seems to be a very interesting aspect as far as the action of α-Sarcin on tumor tissue is concerned.

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