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SHORT COMMUNICATION

Nicotine Affects Behaviour, Morphology and Cortical Cytoskeleton of *Amoeba proteus*

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Summary. Nicotine, a natural alkaloid of tobacco leaves and roots, is the main factor for human tobacco addiction. To assess its toxic effect on motile cells we have made series of experiments on free-living *Amoeba proteus*. It was found that nicotine changed cell morphology, inhibited locomotion, as well it led to degradation of actin cytoskeleton and finally to cell destruction. Obtained results suggest necrosis not the apoptosis as the mechanism of cell death, however many phenomena concerning structure of cytoskeleton and physical cell disintegration suggest that process might to be active.

Key words: *Amoeba proteus*, cytoskeleton, nicotine.

INTRODUCTION

Nicotine $(C_5H_4N)CH(CH_2)_3N(CH_3)$, a natural alkaloid of tobacco leaves and roots, is the main factor for human tobacco addiction (Dani *et al.* 2001), however other substances present in tobacco smoke may also contribute to the damaging effects of smoking. The result of tobacco addiction, long-term cigarette smoking, is a leading cause of premature death because of its contribution to the development of clinical cardiovascular disease and cancer, and is probably one of the most important public health issue of our time. That is why a

great number of laboratories examine the effect of nicotine on living organisms and try to clear up the molecular bases of its activity.

The lipophilic nature of nicotine results in high solubility in membrane lipids and fast influx into cells. It is easily absorbed in human airways and can affect epithelial and blood cells. It was found that tobacco smoke may affect the function of the immune system, first of all the leukocyte cells. Polymorphonuclear leukocytes are the first line of the host defence against acute bacterial infections.

Stimulated leukocytes are highly motile and capable of pseudopodia formation and cytoplasmic flow. In the case of infection they migrate along the chemotactic gradient at a rate of 10-15 μm/min to the site of inflammation, where they phagocytose bacteria and dead cells remnants. Both migration and phagocytosis

require the ability for efficient and properly regulated reorganization of the cytoskeleton.

The effect of tobacco smoke components and nicotine solution on the morphology and function of leukocytes has been described by many authors (i.e., Eichel and Shahrik 1969, Nouri-Shirazi and Guinet 2003, Sikora *et al.* 2003). Oral leukocytes are immediately affected by toxic agents from cigarettes. Leukocytes treated with nicotine (pure nicotine and tobacco smoke) rounded up, lost the ability to form pseudopods and ceased migration (Eichel and Shahrik 1969), however they remained strongly attached to the surface (Blue and Janoff 1978, Corberand *et al.* 1980). Their cytoplasm became motionless and cellular organelles exhibited only Brownian movement (Eichel and Shahrik 1969). As a result, phenomena which are related to cell motility, i. e. the chemotactic response (Bridges *et al.* 1977) and phagocytosis (Blue and Janoff 1978, Ryder 1994) were also disturbed.

It was also found that nicotine inhibits apoptosis, turning off well known mechanisms of protection against pathological changes in cells (Wright *et al.* 1993, Maneckjee and Minna 1994, Hakki *et al.* 2001).

Leukocytes crawl using a common mechanism of locomotion, frequent for other animal tissue cells (e.g. embryonic cells, lymphocytes, invasive tumour cells) and the only form of locomotion for free-living amoebae. This type of cells, contrary to fibroblasts or keratinocytes, does not form tension-loaded cytoskeletal structures. Unfortunately, most of the tissue cells which normally migrate by means of amoeboid movement have to be studied in primary cultures, since their immortal cell lines are non-motile. Transformation strongly changes cell physiology, and the organization of the cell cytoskeleton, which influences the cell's response to toxins. The evident similarity of locomotion of leukocytes and amoebae as well as data concerning morphological and cytological changes in leukocytes treated with nicotine stimulated us to examine the effect of this alkaloid on *Amoeba proteus* cells.

A. proteus is known for years as a perfect model for examination of cell motility (Grębecki 1994, 2001a, b) because of its size and the rate of migration. Both cells move relatively fast, and cover roughly a cell length distance in a minute (300 μm/min for *A. proteus*, 10-15 μm/min for leukocytes) (Bray 1992).

The purpose of this study was to determine the effect of nicotine solution on the morphology, locomotion, adhesion, and actin cytoskeleton localization of *A. proteus*.

Finally we tried to define the nature of changes observed in amoebae after treatment with nicotine; trying to determine whether they are necrotic or apoptotic.

MATERIALS AND METHODS

Amoeba proteus of strain A (Jeon and Lorch 1973) was taken for examination. The cells were grown in glass dishes in Pringsheim medium and fed twice a week with *Tetrahymena pyriformis*. Experiments were performed at room temperature on the third day after feeding. Amoebae were treated with nicotine at a final concentration of 10^{-3} M (Sigma Aldrich). After addition of nicotine the cells were observed for 10 to 60 min under a PZO Biolar microscope equipped with Pluta Differential Interference Contrast (DIC) and images were recorded using a C2400 Hamamatsu camera system and NV 8051 Panasonic time-lapse recorder. For scanning electron microscopic (SEM) observations the cells were fixed in 3.5 % paraformaldehyde, dehydrated through a graded series of ethanol and acetone, dried by the CO_2 critical point technique and coated with gold. SEM observations were performed in a Jeol 1200 EX transmission electron microscope with an ASID 19 scanning attachment, operating at 80 KV.

Fluorescence microscopy was used for studies of actin distribution. *Amoeba proteus* cells were fixed in 3.5% paraformaldehyde solution and then actin filaments were stained with 1% FITC conjugated phalloidin (Sigma-Aldrich). Specimens were observed with a Nikon Diaphot inverted microscope using ×10 0.26NA and ×20 0.4NA Nomarski DIC objectives. Images were collected with a Retiga 1300 (QImaging Inc.) cooled CCD digital camera and transmitted *via* a FireWire interface to a PC computer. For image acquisition and processing AQM Advance 6.0 software (Kinetic Imaging plc) was used.

The cell viability was measured with propidium iodide (Sigma-Aldrich) according to Bielak-Żmijewska *et al.* 2000. To establish the nature of cell death DNA fragmentation test was performed as described by Bielak-Żmijewska *et al.* (2000) and nuclear morphology was studied on cells stained with Hoechst 33258 (Sigma-Aldrich) (concentration 10^{-3}M) in fixed cells (Bielak-Żmijewska *et al.* 2000).

RESULTS

Locomoting *Amoeba proteus* cells were treated with nicotine solution at a final concentration of 10^{-3} M. This concentration was chosen after a series of preliminary experiments because it was the lowest concentration that evoked morphological changes (data not shown). The cells responded to the alkaloid immediately after treatment. They rapidly contracted, lost polarity and detached from the substratum (Figs 1 A-D). As seen on Fig. 2A, the surface of nicotine-treated *A. proteus* is destitute of any adhesive structures (minipodia or rosette-contacts), that are normally present on the ventral surface of untreated migrating amoebae (Fig. 2B, ar-
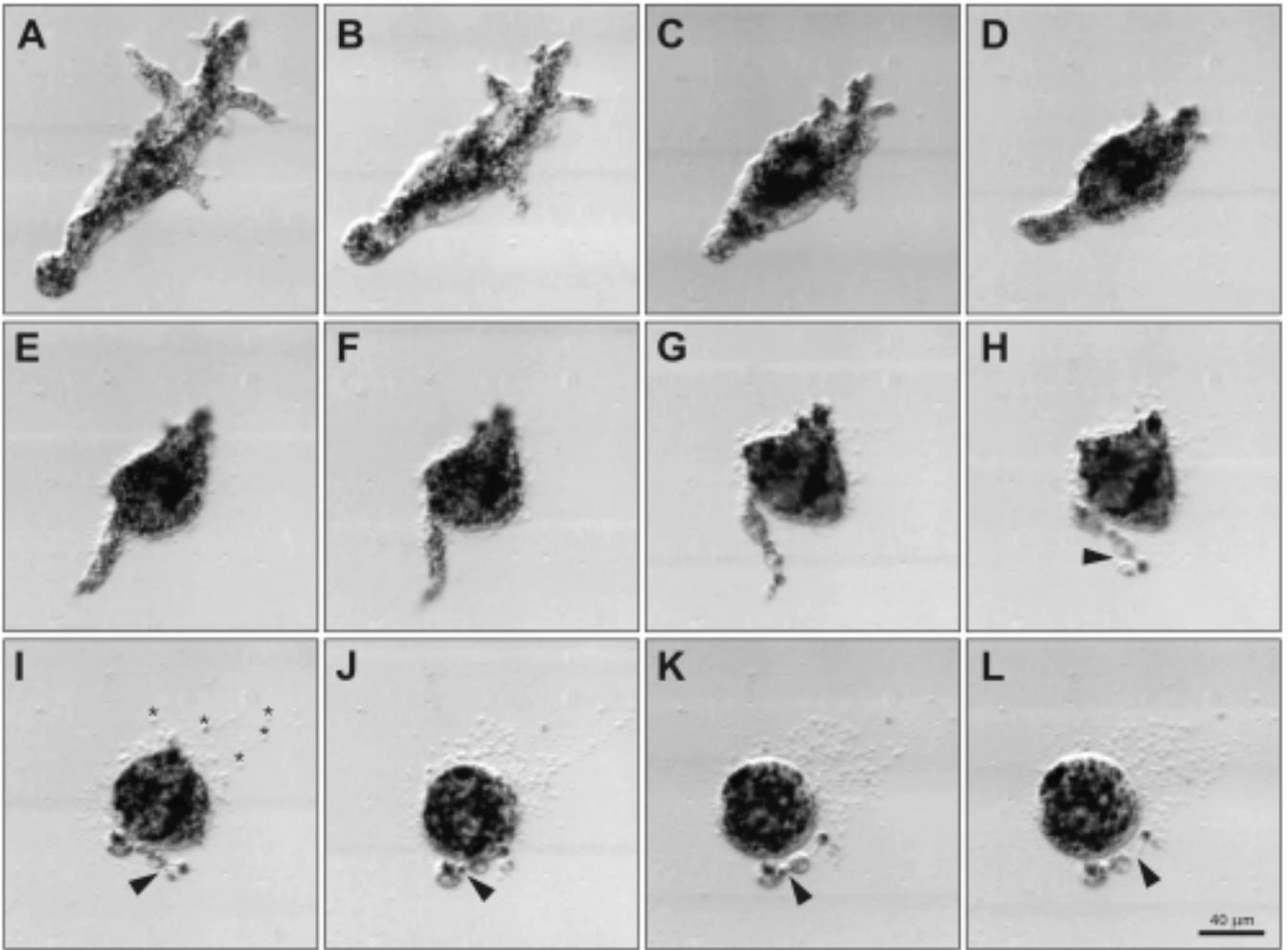
Figs 1 A-L. Consecutive stages (DIC records from 0 to 30 min) of changes evoked by nicotine action on *Amoeba proteus*. arrowhead - site of autotomy; asterisks - blebs separation.
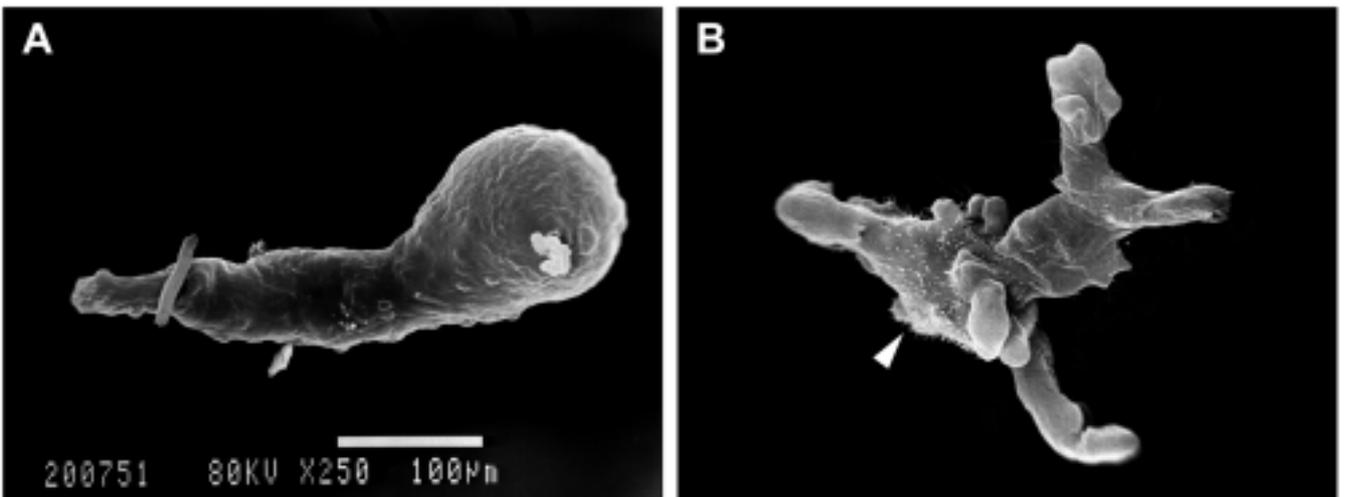

Fig. 2. *A. proteus* treated with nicotine solution (A) and untreated cell (B), arrowhead shows adhesive minipodia on cell surface.
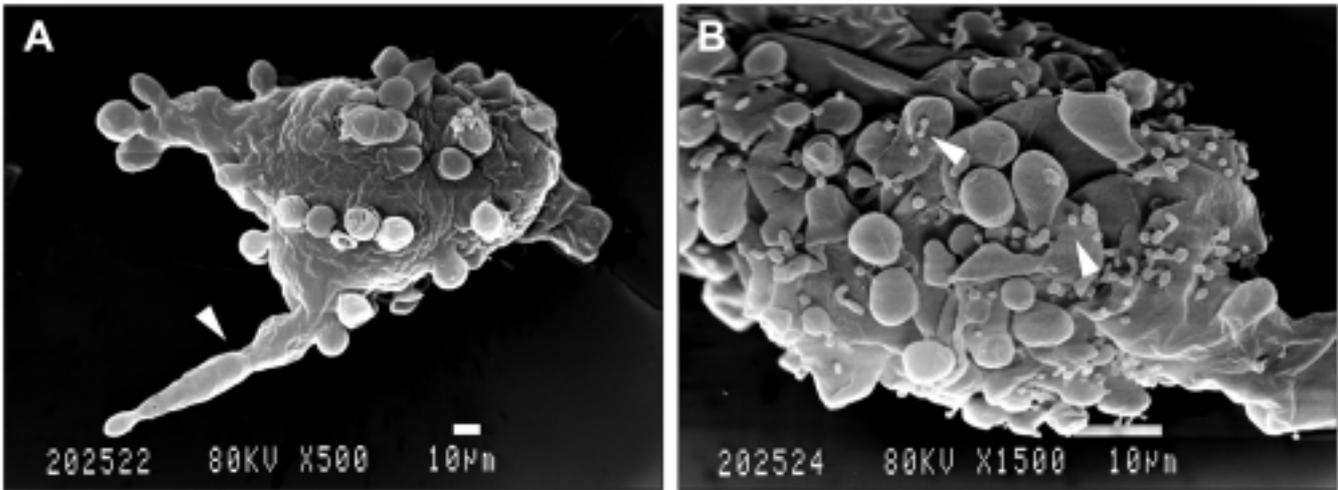
Fig. 3. *A. proteus* with intensive blebbing and long protrusion (A - arrowhead) and small blebs on bleb surface (B - arrowheads).

Fig. 4. Autotomic blebs on amoeba surface.

Fig. 5. F-actin distribution in *A. proteus* treated with nicotine. **A** - phalloidin staining; **B** - Nomarski DIC image (arrowheads - F-actin concentrated in a form of contractile ring).
Fig. 6. F-actin in blebbing *A. proteus*. **A** - phalloidin staining; **B** - Nomarski DIC image (arrowheads - zone of blebs formation).

rowhead shows minipodia). During the first stage of reaction a great variety of cell shapes were formed.

Immediately after treatment amoebae undergo intense blebbing (Fig. 3A), sometimes leading to formation of secondary blebs on the bleb surfaces (Fig. 3B, arrowheads). Some of the cells form large, elongated projections (Figs 1 E-G, 3A, arrowhead). Finally cell contraction leads to autotomy (Figs 1 H-L, site of autotomy marked by arrowhead). Blebs gradually separate and form numerous small vesicles (Figs 1I, asterisks; 4). The irreversibility of these processes means that exposure of *A. proteus* to this nicotine solution leads to the total destruction of the cell.
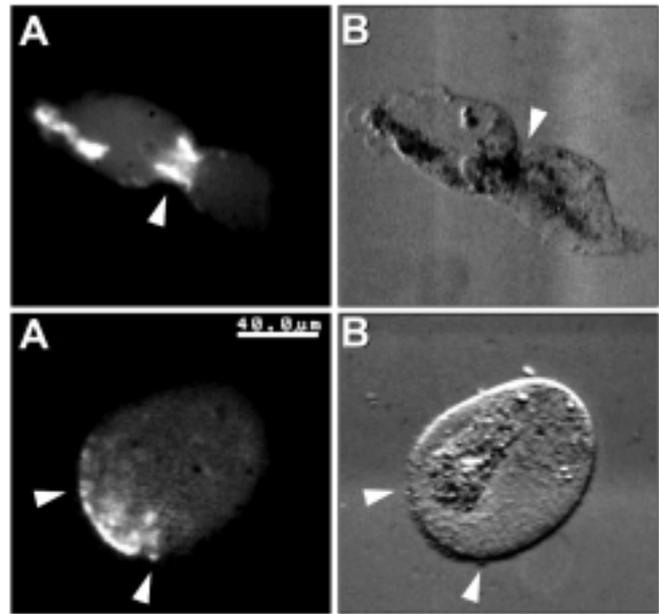
Staining of F-actin with fluorescently labelled phalloidin in amoebae treated with nicotine solution shows that the actin cytoskeleton is concentrated at sites of autotomy, forming structures similar to the contractile ring of a dividing cell (Fig. 5, arrowhead). In blebbing cells actin filaments were localized in areas underlying zones of bleb formation (Fig. 6, arrowheads).

The final result of treatment of *A. proteus* with nicotine solution was cell death. We have performed a series of experiments to determine the character of this process. *In vivo* propidium iodide staining showed that the membrane became perforated in nicotine, the usual symptom of cell death. Since nicotine treatment is said to significantly influence apoptotic death of tissue cells, we decided to check whether the death of nicotine-treated *A. proteus* is apoptotic or necrotic. We have not noticed any differences in morphology of nuclei between control and treated cells and no DNA fragmentation was seen in examined cells. These results suggest that nicotine-induced cell death is necrotic in character.

DISCUSSION

It is well known that nicotine caused changes in the development and function of the human immune system (Buisson and Bertrand 2002, Middlebrook *et al.* 2002, Nouri-Shirazi and Guinet 2003) and leads to tumour development. It modulated the microcirculation, cell proliferation, membrane transport, metabolism of cyto-toxic drugs and hormonal milieu in mammals (Berger and Zelle 1988). It was also found that the ability of cells to enter apoptosis is influenced by nicotine. Some authors postulated (Maneckjee and Minna 1994, Hakki *et al.* 2001) that this alkaloid inhibits apoptotic death of pathologically disordered cells preventing their elimination, whilst others (Berger and Zeller 1988, Yamamura *et al.* 1998) claim the opposite, finding nicotine to be an apoptosis promotor.

Amoeba proteus cells exposed to nicotine solution undergo a series of behavioural and morphological changes: cell contraction, formation of elongated protrusions and intensive blebbing, which finally lead to the total destruction of the cells.

The process of blebbing is often observed as a consequence of the action of various extracellular factors. Blebs are hemispherical protrusions at the cell surface, usually forming very fast and characterized by a lack of organelles within them. Blebbing was observed in various cells including Walker 256 carcinosarcoma cells (Keller *et al.* 2002) or fibroblasts (Pletushkina *et al.* 2001). We have observed a similar response in *A. proteus* cells under the influence of nicotine. Harris (1990) suggested that blebs in tissue cells appeared as the consequence of plasma membrane detachment from the cortical actin layer. He also proposed that later

F-actin may repolymerize under the plasma membrane, permitting bleb retraction. In *A. proteus*, blebbing is irreversible and actin concentrates in the bleb base, promoting vesicle detachment. Irreversible blebbing followed by vesicle detachment may lead to cell membrane loss and may be at least partially responsible for the strong toxic effect of nicotine on *A. proteus*, leading to the cell death.

It is known that the process of blebs formation was also noticed physiologically in locomoting cells (Harris 1990, Keller 2000), during mitosis (Laster and MacKenzie 1996) and apoptosis (Laster and MacKenzie 1996). The complexity of this common process was summarized by Keller *et al.* (2002) "It can be induced by compromising actin polymerization (Harris 1990, Cunningham 1995) or cross-linking of actin filaments (Cunningham 1995, Janmey *et al.* 1992), by disassembly of microtubules (Keller and Zimmermann 1986, Keller and Eggli 1998, Pletushkina *et al.* 2001), by deformation or suction pressure (Schütz and Keller 1998), or by lysophosphatidic acid, which induces phosphorylation of myosin light chains through activation of Rho (Hagmann *et al.* 1999)". Bleb localization might correspond to the sites of adhesive structures observed in locomoting cells. Their appearance in these places may be connected to the uneven distribution of actin prior to the experiment (Grębecka *et al.* 1997). It might to be true only for the first steps of the process because later stages of autotomy are very fast and difficult to study by means of immunocytochemistry.

We hope that this image of active cell death will contribute to a better understanding of the response of the body's immune system to nicotine. This may be especially important for leukocytes from the oral cavity, the first component of the immune system coming into contact with nicotine in the smoker's body, which may also undergo strong morphological changes. The experiments performed suggest necrosis rather than apoptosis as the mechanism of the actual cell death, although many phenomena concerning the structure of the cytoskeleton and physical disintegration of the cell suggest that the process might be active. The best interpretation we can propose for the fate of amoebae after nicotine application is deregulation of the cell motility machinery in such a way that, in spite of propelling cell movement, its modified and intense contractile activity causes cell disintegration and death.

Even if the results obtained do not lead to an optimistic conclusion concerning the possibility to diminish toxic

activity of nicotine in the smoker's body, a deeper understanding of its toxicity can be an argument against using any form of tobacco.

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