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Effect of *Clostridium perfringens* epsilon toxin on MDCK cells

Erika Borrmann *, Horst Günther, Heike Köhler

Federal Institute for Health Protection of Consumers and Veterinary Medicine, Division 4, Jena, Germany

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Abstract

Epsilon toxin is one of the major lethal toxins produced by *Clostridium perfringens* type D and B. It is responsible for a rapidly fatal disease in sheep and other farm animals. Many facts have been published about the physical properties and the biological activities of the toxin, but the molecular mechanism of the action inside the cells remains unclear. We have found that the *C. perfringens* epsilon toxin caused a significant decrease of the cell numbers and a significant enlargement of the mean cell volume of MDCK cells. The flow cytometric analysis of DNA content revealed the elongation of the S phase and to a smaller extent of the G2+M phase of toxin-treated MDCK cells in comparison to untreated MDCK cells. The results of ultrastructural studies showed that the mitosis is disturbed and blocked at a very early stage, and confirmed the toxin influence on the cell cycle of MDCK cells.

Keywords: Epsilon toxin; Madin–Darby canine kidney cell; Cell cycle; Flow cytometry; Electron microscopy; *Clostridium perfringens* type D

1. Introduction

Clostridium perfringens type D is the causative organism of the rapidly fatal enterotoxaemia in sheep, goats, calves and, occasionally, other species. The enterotoxaemia known as sudden death, pulp kidney or overeating disease is characterised by oedema in lung, kidney, heart and brain [1,2]. The major lethal toxin is the epsilon toxin produced as inactive protoxin (311 amino acids) with a molecular mass of 32.7 kDa [3]. The protoxin is converted by proteolytic enzymes to the fully active toxin with the loss of an N-terminal protein (13 amino acids) [4,5]. The activated toxin possesses lethal, dermonecrotic and oedematic activities [6]. Small amounts of epsilon toxin in the gut of normal animals are considered innocuous, while a higher concentration of the toxin as a result of sudden changes to rich diet increases the permeability of the intestine, leading to the absorption of large amounts of toxin into the systemic circulation [1,7]. The absorbed toxin increases the vascular permeability, causes severe vascular damage and can pass the blood–brain barrier. The permeabilisation of blood vessels is a consequence of interaction between the epsilon toxin and a receptor on vascular endothelial cells. A sialoglycoprotein located in synaptosomal membranes is discussed as a high-affinity binding site [8].

Ultrastructural studies of the brain tissues revealed that tight junctions in the vascular endothelium break down [9]. The *etx* gene encoding epsilon toxin has been isolated [6]. Many facts have been published about the physical properties and the biological activities. Recently the toxin was reported to be cytotoxic for Madin–Darby canine kidney (MDCK) cells through formation of a large membrane complex, but the nature of toxin action on cells remains unclear [3,5,10]. Recently a novel virulence mechanism of pathogenic bacteria that targets the cell cycle was discussed in connection with the cytolethal distending toxin produced by diarrhoeal disease-causing enteropathogens [11,12]. The cell cycle-modulating properties of different bacterial proteins have been known for years [13–15]. In addition, a variety of toxins secreted by certain species of *Clostridium* bacteria have been discovered to covalently modify and inactivate several members of the Rho family of signal-transducing small GTPases [16]. Rho GTPases act as molecular switches in diverse signal transduction processes. They are involved in the organisation of the actin cytoskeleton as well as in cell cycle progression [17,18]. Eventually, the epsilon toxin could change the cell cycle progression via the modification of Rho proteins.

* Corresponding author. Tel.: +49 (3641) 804-251; Fax: +49 (3641) 804-228.

E-mail address: e.borrmann@bgvv.de (E. Borrmann).

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Cell proliferation defined as the increase in cell number resulting from completion of the cell cycle is an attractive endpoint for 'in vitro toxicity assays', since nearly any kind of damage in cells may result in altered cell proliferation [19]. Flow cytometry makes it possible to investigate cell proliferation via DNA analysis on the single cell level.

In this study, we investigate (1) the influence of the toxin on the cell proliferation (cell cycle) of MDCK cells and (2) the morphological alterations of MDCK cells in contact with the epsilon toxin, in order to obtain more information about the mode of the toxin action on eukaryotic cells.

2. Materials and methods

2.1. Toxin

The epsilon toxin was produced by cultivation of *C. perfringens* type D, NC 0834640, in reinforced clostridial medium at 37°C for 18 h, anaerobically, using fermenter, harvested and centrifuged at 15 000 × *g* for 20 min at 4°C. The culture filtrate was purified and concentrated by ultrafiltration (5–30 kDa) (ITTH, Georg-August-Universität, Göttingen, Germany). Before the test the epsilon protoxin produced by *C. perfringens* type D was activated to the epsilon toxin by treatment with 0.01% trypsin, 1 h, 37°C.

In preliminary trials the toxin was used in the titre steps 1:5000, 1:8000 and 1:10 000. These titres were also used for the electron microscopic experiments. On the basis of the results of these experiments the following toxin titres were chosen for further investigations: 1:6000, 1:7000, 1:8000. The different concentrations of the toxin were prepared by dilution with cell culture medium supplemented with 5% foetal calf serum (FCS).

2.2. Cells

MDCK cells were obtained from the American Type Culture Collection (ATCC, CCl 34), maintained in Eagle's minimal essential medium (MEM) with non-essential amino acids, 10% FCS, 2 mM glutamine, 50 μg ml^{-1} gentamicin and cultivated at 37°C in a humidified incubator with 5% CO_2.

2.3. Electron microscopy

The cells (3×10^5 cells ml^{-1}) were seeded in 25-cm^2 cell culture flasks (Costar GmbH, Germany) using a cell medium with 10% FCS and incubated for 8 h at 37°C to allow adherence. In order to synchronise the cells the medium was replaced by a medium without serum and incubated for further 16 h. Then the flasks were divided into a control group receiving fresh MEM with 5% FCS and three other groups receiving medium containing three different concentrations of epsilon toxin (1:5000, 1:8000, 1:10 000). After 5 h or 24 h incubation at 37°C and 5% CO_2 without or with toxin all incubation media were replaced by fresh cultivation medium for another 24 h.

Then the cells were trypsinised and centrifuged softly. The sediment was fixed with 2.5% glutaraldehyde for 2 h, mixed with agarose, fixed with 2% osmium tetroxide for 1 h and embedded in resin (Durcupan). Thin sections were observed in an electron microscope BS 500 (Tesla, Brno, Czech Republic).

2.4. Assessment of cell numbers and mean cell volumes

The prepared flasks (Section 2.3) were divided into a control group receiving fresh MEM with 5% FCS and three other groups receiving medium containing three different concentrations of epsilon toxin (1:6000, 1:7000, 1:8000). After a 24-h incubation of cells with toxin at 37°C and 5% CO_2, the media were replaced by fresh media (10% FCS) for further incubation except for a control flask and a toxin flask. The number of viable cells and the DNA content were measured after 24 h exposure time of the toxin (day 0), 24 h exposure time+24 h cultivation time with fresh medium (day 1) and 24 h exposure time+48 h cultivation time with fresh medium (day 2). The evaluation of the viability was performed microscopically and by means of the measurement of the cell numbers and mean cell volumes using the cell counter and analyser system CASY 1 (Schärfe System, Reutlingen, Germany). CASY 1 permits the determination of the cell number, the mean cell volume and the total cell volume for quantification of the cytotoxic effect. The basic principle of the volume determination is the measurement of the conductivity of an electrolyte solution. Cells with an intact cell membrane have very low conductivity while damage of the cell membrane leads to an increase in conductivity.

We chose the cell number and the mean cell volume of the cells for the assessment of the toxin influence. In order to estimate the number of cells and the mean cell volumes, cells were rinsed, trypsinised and resuspended in cell culture medium (10% FCS). From this cell suspension a sample was diluted in 10 ml of the electrolyte solution Casyton. The remaining cell suspension was used for flow cytometry.

The cell numbers and the mean cell volumes were measured in three independent tests. The means and S.E.M. were calculated.

2.5. Flow cytometric assessment of the MDCK cell cycle

The preparation of the cell suspension and the staining procedure with propidium iodide were performed according to the instruction of the Cycle Test™ Plus DNA reagent kit (Becton-Dickinson, San Jose, CA, USA). The cell suspension was centrifuged for 5 min at 300 × *g* and the cells were resuspended in a buffer solution containing sodium citrate, sucrose and dimethyl sulfoxide. For dis-

solving the cell membrane lipids, eliminating the cell cytoskeleton and nuclear proteins a trypsin buffer was added. After incubation for 10 min a trypsin inhibitor and ribonuclease A for digesting the cellular RNA were also added. The staining procedure was carried out using the propidium iodide solution. The flow cytometric analysis was performed on a Becton-Dickinson FACScan fitted with an argon ion laser tuned to 488 nm at a power output of 15 mW. 10 000–20 000 cells were recorded. The data were analysed with the DNA software package supplied by Becton-Dickinson. Flow cytometry analysis of DNA content was repeated in three independent tests.

2.6. Statistical analysis

Cell numbers, cell volume and the percent of cells in the different stages of the cell cycle were expressed as the mean of different tests ± S.E.M. Significant differences were calculated using the paired Student's *t*-test with $\alpha < 0.01$ being considered to indicate statistical significance.

3. Results

3.1. Evaluation of the viability of the MDCK cells

The viability of MDCK cells was measured from day 0 to day 2 after exposure to epsilon toxin for 24 h by means of the determination of the cell numbers and the mean cell volumes. Microscopic examination showed that the morphological changes of the cells were characterised by spindle-like, enlarged cells and single round cells. The cell numbers assessed at 24 h after the initial exposure to the toxin were reduced with increasing concentration (Fig. 1). The differences between control cells and cells with toxin contact were significant. After the medium changes the cells were able to regrow and the cell number increased but the cell numbers in the toxin-treated samples were always lower than the cell number of the control. The differences in the cell numbers between control cells and toxin-treated cells were significant for all toxin concentrations.

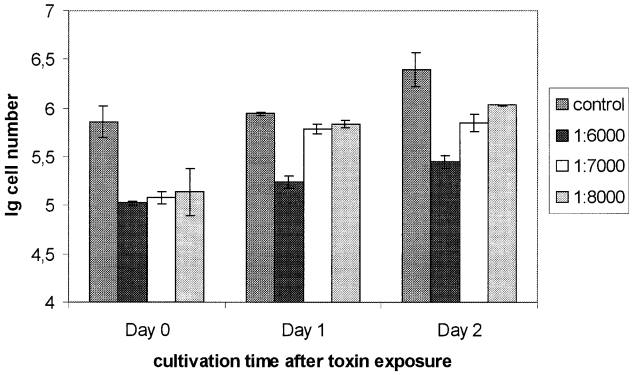

Fig. 1. Toxin concentration- and time-dependent changes in cell numbers of MDCK cells.

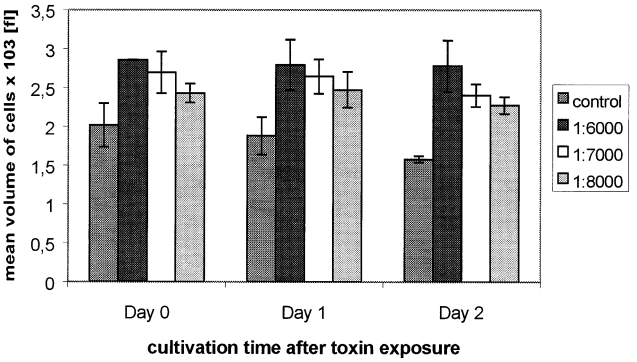

Fig. 2. Toxin concentration- and time-dependent changes in mean cell volume of MDCK cells.

An increase in the mean volumes of the toxin-treated cells in comparison to the control cells depending on the toxin concentration was found (Fig. 2). The higher the toxin concentration, the stronger the increase of the mean cell volume. The increase of the mean cell volume was significant with the exception of the 1:8000 toxin concentration on day 0. On the other days the differences between the mean cell volumes of toxin-treated cells and of control cells were significant ($\alpha < 0{,}01$).

3.2. Evaluation of the MDCK cell cycle and DNA synthesis

The ability of epsilon toxin to influence the cell cycle of the MDCK cells was tested by analysing the DNA content using flow cytometry. The changes in the cell cycle caused by epsilon toxin are shown in Table 1. On all days after toxin treatment there were significant differences in the cell cycle distribution between toxin-treated cells and control cells (cells cultivated without toxin). The proportion of treated cells in the G1 phase was always lower than that of untreated cells, especially on day 2. For the cell control the G1 phase population was 81.3% while for toxin-treated cells the G1 population amounted to 30% for the lowest toxin concentration and 22.5% for the highest concentration. The proportion of cells in the S phase rose markedly caused by the epsilon toxin. On the first day after toxin exposure (day 0) 22% of the untreated cells but 34–37% of the treated cells were in the S phase. After 24 h incubation in cell medium (day 1) about 55% of the treated cells rested in the S phase in comparison to 26% of the control cells. After a further 24 h incubation in cell culture medium (day 2) the proportion of control cells in the S phase decreased to 7.4%, but the proportion of treated cells was 53–60%. The control cells continued to proliferate during the experiment, while the cell proliferation of the toxin-treated cells was markedly reduced.

3.3. Ultrastructural changes

The changes in the course of cultivation time caused by the toxin are summarised in Table 2. During cultivation there was an increase in the size of the cells and the num-

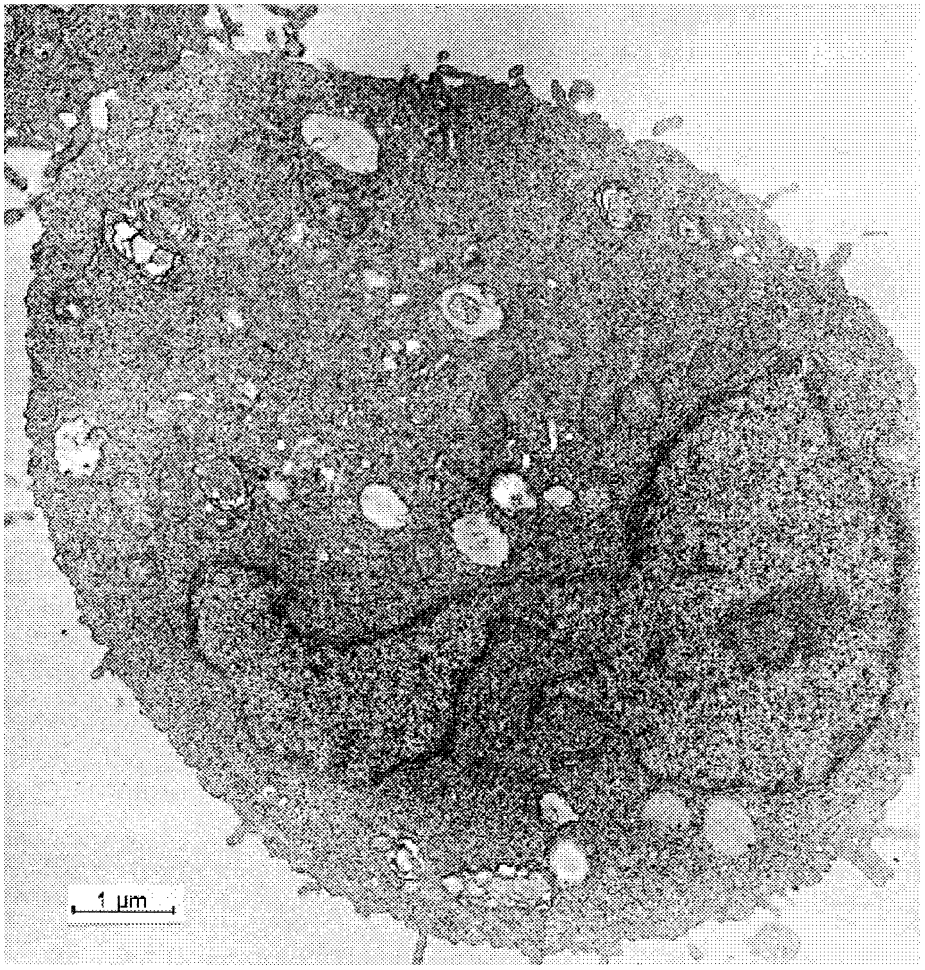

Fig. 3. Control cell. Large nucleus with marginated chromatin, well-developed nucleolus, and some small membrane-limited vacuoles and phagosomes.

ber of mitochondria and phagosomes. In the control cultures all larger, i.e. not only peripherically cut, cells showed a nucleus with an accumulation of dense heterochromatin under the nucleus membrane, and with large prominent nucleoli (Fig. 3). The density of the remaining caryoplasm was lower than that of the cytoplasm. The toxin caused an increase of the diameter of the large cells. The number of denucleated cells as well as swelling or condensation of mitochondria and lysis of the membrane surrounding phagosomes (Fig. 4) clearly increased depending on the concentration of the toxin but independent of the time of effect. The nuclei were in general denser, the heterochromatin rather scattered and the nucleoli more difficult to spot (Fig. 5). After treatment with higher concentrations of the toxin for 5 h, especially many cells were in disintegration, characterised above all by loss of ribo-

Table 1
Cell cycle distribution of MDCK cells after epsilon toxin contact for 24 h

Day after toxin contact	% of cells in cell cycle phase (mean ± S.E.M.; $n = 3$)			
	System	G1	S	G2+M
0	control	53.3 ± 10.2	22.4 ± 6.0	24.3 ± 3.7
	1:8000	41.7 ± 4.1	34.2 ± 6.0	24.1 ± 1.7
	1:7000	36.8 ± 1.5	37.0 ± 3.0	26.2 ± 4.6
	1:6000	39 ± 0.9	35.5 ± 1.0	24.5 ± 1.3
1	control	58.0 ± 4.0	26.0 ± 4.7	16 ± 1.2
	1:8000	23 ± 10.9	55.5 ± 1.7	21.6 ± 3.8
	1:7000	18.9 ± 6.7	53.6 ± 9.1	27.5 ± 2.4
	1:6000	19.5 ± 6.1	55.5 ± 14.5	25 ± 8.5
2	control	81.3 ± 2.4	7.4 ± 0.7	11.3 ± 1.8
	1:8000	30 ± 0.3	53.4 ± 1.1	16.6 ± 1.0
	1:7000	18.9 ± 4.2	61.9 ± 5.2	19.2 ± 2.8
	1:6000	22.5 ± 2.1	60.5 ± 4.2	17 ± 2.5

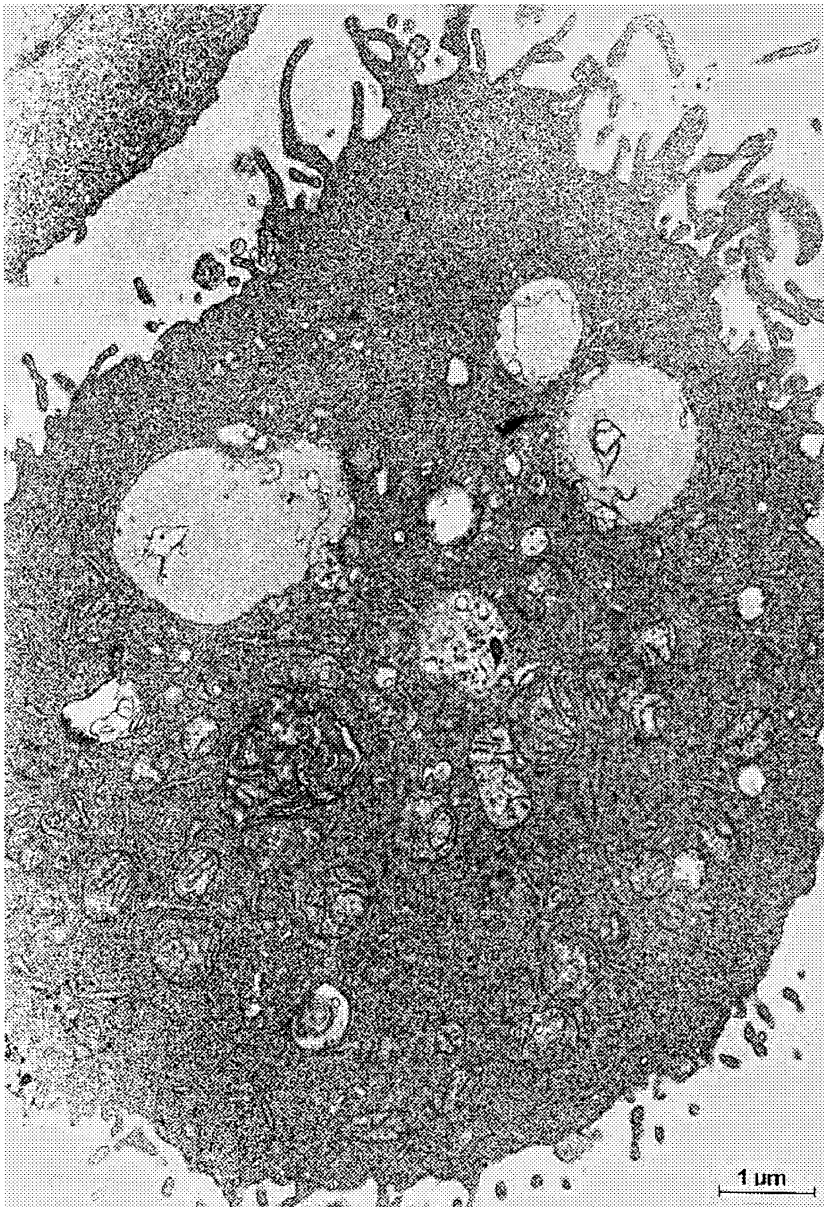

Fig. 4. Effect of toxin 1:5000 after 24 h. Large cell without visible nucleus, swollen mitochondria, limiting membrane of phagolysosomes partly disintegrated.

Table 2
Effect of *C. perfringens* epsilon toxin on MDCK cells

Toxin	Diameter of large cells (µm)	Cells without nucleus	Density of nucleus	Mitochondria		Phagolysosomes	
				Number	Swelling or condensation	Number	Lysis of membrane
Control	10	+	+/++	ca. 12/cell	−	+	−
5 h, 1:5000	15	++++	+++/++++	in denucleated cells	++++	++	+++
1:8000	15	++	+++/++++	markedly less	+	++	+
1:10000	15	+	+++	ca. 16/cell	+	++++	−
Control 24 h later	15	+	+/++	15–20/cell	+	++	−
24 h, 1:5000	17	++ (∼1/3)	+/+++	extremely	++++	+++	++++
1:8000	17	++ (∼1/4)	+/+++	different	+++	++	+
1:10000	18	+	+/+++		++	+	−

Intensity of change: − absent; + slight; ++ moderate; +++ medium; ++++ strong.

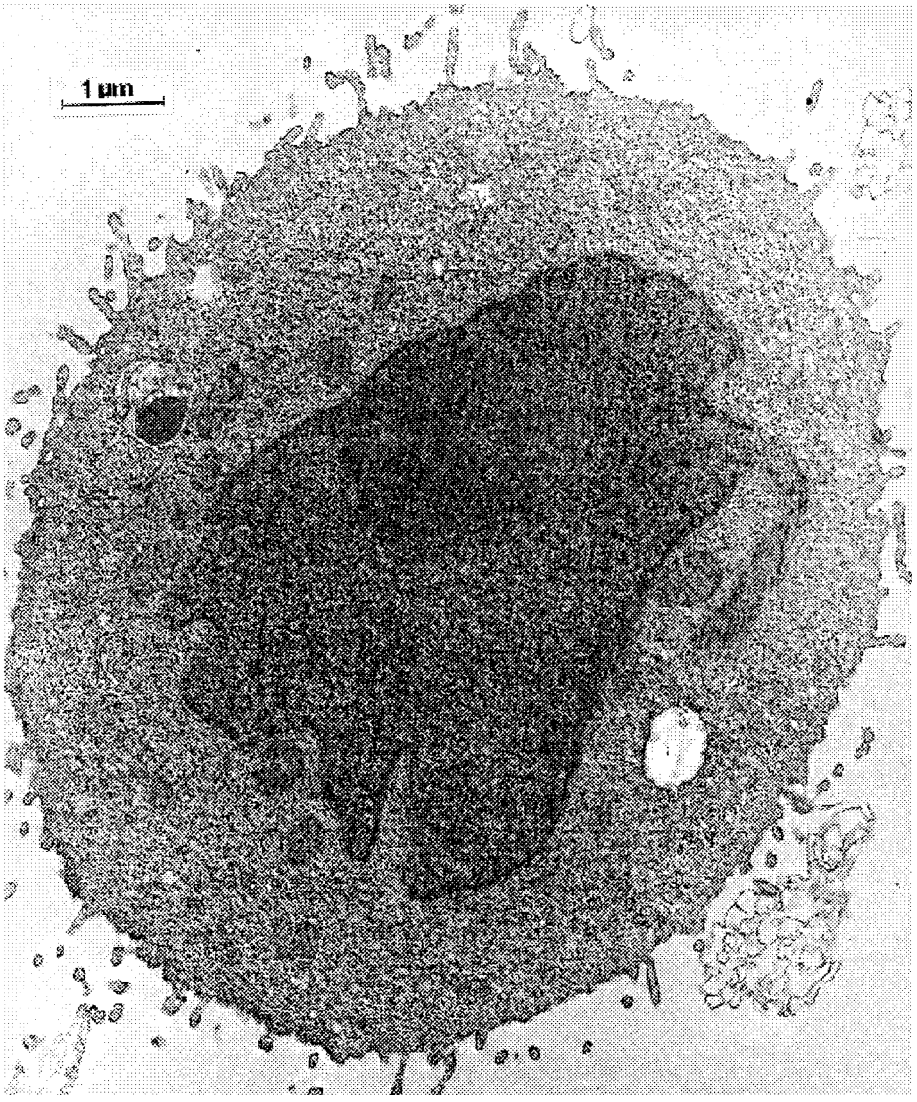

Fig. 5. Effect of toxin 1:8000 after 5 h. Dense nucleus, few organelles.

somes due to membrane defects. The number of mitochondria and phagosomes showed no clear tendency. Indications of apoptosis did not appear, especially no formation of nuclear fragments, no detachment of cell particles, no condensation but swelling of the cytoplasm.

4. Discussion

Several reports have been published on the cytotoxic effect of epsilon toxin on MDCK cells [20–23]. It was shown that the action of epsilon toxin on the MDCK cells was specific. The cytotoxic effect was found to be very rapid and potentiated by EDTA. The rapid cell death suggested that the MDCK cells can express a receptor for the toxin [21]. Recently, it was described that the epsilon toxin cytotoxic activity was correlated with the formation of a large membrane complex and efflux of intracellular K^+ without entry of the toxin into the cytosol [5]. The minimal concentration for cytotoxic activity was found to be 200 ppm [22]. Because we did not use the purified toxin but a concentrated culture filtrate purified by ultrafiltration, at first the suitable toxin concentrations were tested in preliminary experiments. The toxin concentrations were chosen in such a way that the cell proliferation was inhibited but cells were not completely damaged. The electron microscopic investigations were carried out with titres of 1:5000, 1:8000 and 1:10 000. The toxin titre of 1:5000 resulted in a strong decrease of the cell numbers so that there were too few cells for flow cytometric measurement. The toxin titre of 1:10 000 did not show noticeable changes by flow cytometry. Therefore we used toxin titres of 1:6000, 1:7000 and 1:8000 for the flow cytometric assessment. Previously a number of reports have described the ability of bacterial proteins to inhibit or stimulate the cell proliferation of eukaryotic cells [14]. We have shown that the *C. perfringens* epsilon toxin caused a significant decrease in cell numbers, a sig-

nificant enlargement of the mean cell volume and changes in the cell cycle distribution compared to the control cells on all days after a 24-h toxin exposure. The share of cells in the S phase was significantly higher among the toxin-treated cells than among the control cells. As a consequence of the toxin influence on the cell cycle the cells were no longer able to divide and differentiate. This fact explains the decreasing cell numbers depending on the cultivation time (day 1 and day 2). The epsilon toxin in low dose arrested cell division without immediately killing the cells. A part of the cells, however, survived the toxic injury and developed further, but the influence of the toxin concentration was clearly seen. Prolongation of the incubation time over 5 h did not play any role. This result corresponds to the very short effective time reported by Lindsay et al. [10]. The ultrastructural changes indicate a caryotoxic effect of the toxin. It seems that mitosis is disturbed and blocked at a very early stage so that the chromosomes lie irregularly clustered in the nucleus. The condensation of the nuclei, already described by Hambrook et al. [24], indicates a complete inhibition of synthesis activity. The changes of the mitochondria and, finally, their complete disappearance result in the breakdown of the energy supply of the cells. Swelling of cells points to functional disturbances of the cell membrane as described by Petit et al. [5]. Hambrook et al. [24] reported convolution and disruption of membranes. According to our observations many denucleated cells with damages of mitochondria but with cytoplasm rich in ribosomes speak for the rare occurrence of grosser structural defects of the cell membrane. The latter seem to appear at a later stage and could be the consequence of the metabolic disturbance and not of a direct toxic effect. The increased number of membrane-surrounded phagosomes at 29 h after the beginning of toxic challenge already indicates a compensation for defects by the cells. The finding that mitosis is disturbed and blocked at a very early stage agrees with the results of the DNA analysis which have shown the elongation of the S phase and, although to a lesser extent, of the G2+M phase.

Cell cycle-modulating bacterial proteins such as the cytotoxic necrotising factors (CNF) produced by *Escherichia coli* and cytolethal distending toxin produced by a number of enteropathogenic bacteria, which inhibit cell proliferation and eventually result in epithelial cell death, remove the major barrier to entry of pathogenic bacteria into the body [14]. On the other hand, CNF of *E. coli* is also described as a Rho-modifying toxin. It is known that the epsilon toxin increases the intestinal permeability with subsequent absorption of the toxin into the circulation [1]. The first step in this procedure might be the disturbance of the cell cycle of the intestinal epithelial cells by the action of the epsilon toxin already at a low dose. But this hypothesis has to be proved in further investigations using epithelial cells.

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