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Oligomycin, inhibitor of the F_0 part of H^+-ATP-synthase, suppresses the TNF-induced apoptosis

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The release of cytochrome *c* from the intermembrane space of mitochondria into the cytosol is one of the critical events in apoptotic cell death. In the present study, it is shown that release of cytochrome *c* and apoptosis induced by tumor necrosis factor α (TNF) in HeLa cells can be inhibited by (i) overexpression of an oncoprotein Bcl-2, (ii) Cyclosporin A, an inhibitor of the mitochondrial permeability transition pore (PTP) or (iii) oligomycin, an inhibitor of H^+- ATP-synthase. Staurosporine-induced apoptosis is sensitive to Bcl-2 but insensitive to Cyclosporin A and oligomycin. The effect of oligomycin is not due to changes in mitochondrial membrane potential or to inhibition of ATP synthesis/hydrolysis since (a) uncouplers (CCCP, DNP) which discharge the membrane potential fail to abolish the protective action of oligomycin and (b) aurovertin B (another inhibitor of H^+-ATP-synthase, affecting its F_1 component) do not affect apoptosis. A role of oligomycin-sensitive F_0 component of H^+-ATP-synthase in the TNF-induced PTP opening and apoptosis is suggested.
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Introduction

Mitochondria play a key role in the commitment of cells to apoptosis induced by cytokines, hormones and other natural regulators as well as by reactive oxygen species (ROS) (reviewed in Skulachev, 1996a, 1998; Kroemer and Reed, 2000). They are the target for action of pro- and anti-apoptotic proteins of the oncoprotein Bcl-2 family, some other proteins, including p53, and low molecular weight mediators of apoptosis (ceramide, Ca^{2+}, etc.). The main response of mitochondria to apoptotic stimuli is the release of a number of soluble apoptosis-inducing proteins from the intermembrane space into the cytosol. Among them are cytochrome *c* (Yang *et al.*, 1997; Kluck *et al.*, 1997), apoptosis inducing factor (AIF) (Kroemer *et al.*, 1998), procaspases-2, -3, -9 (Samali *et al.*, 1999), Smac/Diablo (an activator of caspases, which blocks the action of natural inhibitors of caspases) (Du *et al.*, 2000; Verhagen *et al.*, 2000) and endonuclease G (Li *et al.*, 2001). Overproduction of ROS in mitochondria, which is usually regarded as their bioenergetic malfunctioning, may, in fact, play a role as a mechanism multiplying or even generating apoptotic signals (reviewed in Skulachev, 1996b, 1999).

The molecular mechanism of cytochrome *c* release from mitochondria remains a most intriguing enigma. At least in some cases of apoptosis, the PTP opening is involved in this process (Kroemer, 1997). The PTP opening entails a high amplitude osmotic swelling of the mitochondrial matrix and, as a consequence, disruption of the outer mitochondrial membrane (Skulachev, 1996a). It is also possible that temporary opening of the PTP results in minimal swelling and changes in topology of the intermembrane compartment which might be critical for cytochrome *c* release caused by permeabilization of the outer membrane with Bax, Bak, Bid, etc. (Scorrano *et al.*, 2002).

Apoptosis in some cells can be triggered by mitochondrial inhibitors through the reduction of ATP level (Wolvetang *et al.*, 1994). Oligomycin and some analogous antibiotics (apoptolidin, ossamycin and cytovaricin) which are known to inhibit mitochondrial H^+-ATP-synthase were shown to be among the top 0.1% most cancer cell selective cytotoxic agents (37 000 compounds were tested on the 60 human cancer cell lines collected at the National Cancer Institute, USA) (Salomon *et al.*, 2000). In less sensitive cells, the inhibitors of glycolytic ATP production promoted their effect. Reduction of ATP can also block apoptosis and/or switch apoptotic death mechanism to necrosis. This effect was assumed to underlay an anti-apoptotic action of oligomycin during apoptosis induced by such anticancer drugs as etoposide and dexamethasone (Eguchi *et al.*, 1997; Leist *et al.*, 1997). Other mechanisms of anti-apoptotic action of oligomycin have also been suggested. It was reported to block dimerization of Bax (a pro-apoptotic Bcl-2 family member) and following cytochrome *c* release involved in the calphostin C-induced apoptosis (Ikemoto *et al.*, 2000). Inhibition of cytochrome *c* release by oligomycin was

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also inherent in some Bax-independent apoptoses (Matsuyama *et al*., 1998; Goldstein *et al*., 2000).

In the present study, effects of two inducers of apoptosis were investigated, namely Sts and TNF. Apoptosis induced by Sts, a nonselective inhibitor of protein kinases, resembles the great variety of stress-induced apoptoses. Signaling pathways of this type usually involve mitochondria as well as Bax, which oligomerizes and binds to mitochondria (Hsu *et al*., 1997). The early events in TNF-induced apoptosis include a TNF binding to specific receptors, activation of caspase-8 and cleavage of Bid (one of the 'BH3-only' members of the Bcl-2 family), which is further N-myristoylated and translocated to mitochondria (reviewed in Korsmeyer *et al*., 2000). The following Bid-induced mitochondrial events depend on Bak, a close relative of Bax which, in contrast to Bax, is always bound to the outer mitochondrial membrane (Wei *et al*., 2000). The data on a possible role of the PTP in Sts- and TNF-induced apoptoses are controversial partially because of a complex pattern of accompanying disturbances in mitochondrial energetics, which may differ in different cells. In our experiments, we used HeLa cells. These cells, as was found in our group, are resistant to pro- and antiapoptotic effects of the inhibitors of mitochondrial respiratory chain and uncouplers of oxidative phosphorylation (Shchepina *et al*., 2002). It is found that oligomycin arrests the TNF-, but not Sts- induced apoptosis.

Results

Inhibitors of respiration, uncouplers and oligomycin do not affect Sts-induced apoptosis

To test the effect of inhibitors of oxidative phosphorylation on apoptosis, we choose the HeLa carcinoma cell line. These cells (as a number of other rapidly growing, non-differentiated tumor cell lines) have high levels of both respiration and aerobic glycolysis. The inhibitors of H^+-ATP-synthase oligomycin (5 μg/ml) and aurovertin B (10 μM) was shown to strongly suppress, and uncoupler (0.1 mM DNP) stimulates, the cell respiration, indicating that it is tightly coupled to ATP synthesis (Figure 1). Inhibitors of respiration, oligomycin and uncouplers did not arrest proliferation of HeLa cells for at least 24 h in the presence of glucose. 2-deoxyglucose (DOG, 5 mM), a competitive inhibitor of glycolytic ATP production, was also non-toxic but caused necrotic cell death in combination with any mitochondrial inhibitor mentioned (Figure 2a). In this case, no signs of apoptosis were detected and Bcl-2 did not protect against the cell death (not shown). These data indicate that the mitochondrial and glycolytic ATP productions are interchangable for survival of HeLa cells.

Sts caused rapid morphological changes in HeLa cells (80% cells in 8 h), appearance of blebs on their surface, and condensation and fragmentation of chromatin, which are characteristic for apoptosis (not shown). Oligomycin, respiratory inhibitors and uncouplers did not affect apoptotic cell death and the release of cytochrome *c* from mitochondria into cytosol. Overexpression of Bcl-2 in HeLa cells prevented the release of cytochrome *c* and cell death induced by Sts. These effects of Bcl-2 were insensitive to mitochondrial inhibitors (Figures 2b and 3c). Measurements of oxygen consumption showed that expression of Bcl-2 did not change effects of oligomycin, uncouplers and respiratory inhibitors on the respiration rate (Figure 1).

TNF-induced apoptosis is insensitive to inhibitors of respiration and uncouplers but is suppressed by oligomycin

TNF is shown to induce typical apoptosis in HeLa cells only in combination with emetine, inhibitor of protein synthesis (Sidoti-de Fraisse *et al*., 1998). We confirmed this observation and found no necrotic death during 8–15 h TNF treatment. Apoptotic changes in cellular morphology and condensation of chromatin were observed. They correlated with the cytochrome *c* release into cytosol and were prevented by Bcl-2. The inhibitors of respiration and the uncouplers did not interfere with cytochrome *c* release, apoptosis and the protective action of Bcl-2 (Figures 2c and 3).

In contrast to the effects of Sts, the TNF-induced release of cytochrome *c* and apoptosis were strongly inhibited by oligomycin (Figures 2c and 3). Na^+, K^+-ATPase in the plasma membrane, the only known non-mitochondrial target of oligomycin, was not responsible for this effect since ouabain (0.5 mM), the specific inhibitor of this enzyme, was ineffective in suppressing the TNF-induced apoptosis (not shown). Oligomycin inhibited apoptosis even when it was added 1 or 2 h after TNF but became ineffective after the 3 h TNF treatment (Figure 2d). These data indicate that oligomycin inhibits the TNF signaling after formation of the death-inducing-signaling complex (DISC) composed of the TNF receptor, adapter proteins and procaspase-8. On the other hand, the late steps of the apoptotic cascade (presumably after the release of cytochrome *c*) were unaffected by oligomycin. It should be stressed that oligomycin delays, rather than prevents, apoptosis. The 24 h TNF treatment in the presence of oligomycin was found to result in apoptosis in 90% cells. A similar decline in the protective action of Bcl-2 was also observed (not shown).

Oligomycin was shown to inhibit various TNF-induced responses related to apoptosis. One of the earliest oligomycin-sensitive events observed proved to be the 'marginal' blebbing which occurs at the active lamellar cell edge and is presumably a consequence of disruption of the actin filaments (Domnina *et al*., 2002) (not shown). Overproduction of ROS, detected 3–5 h after the TNF treatment, was also inhibited by oligomycin (Figure 4). Excessive ROS production was observed in various kinds of apoptosis, including TNF-induced apoptosis of HeLa (Sidoti-de Fraisse *et al*., 1998) but the source of ROS was not identified. In our experiments, rotenone and myxothiazole did not inhibit

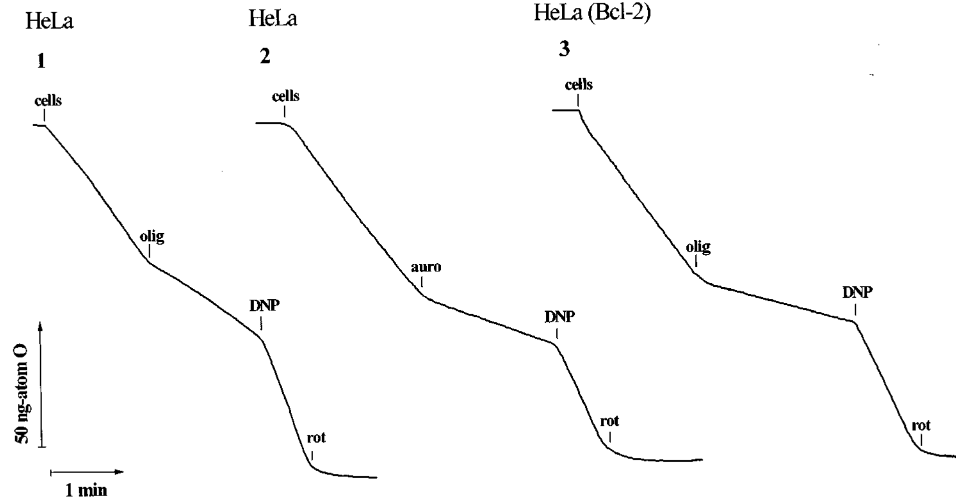


Figure 1 The effect of mitochondrial inhibitors on respiration of HeLa (1,2) and HeLa(Bcl-2) (3) cells. Cells (10^7/ml) were suspended in Hanks Balance Salt medium supplemented with 10 mM glutamine and 30 mM HEPES, pH 7.3, and respiration was measured in the closed chamber at 37°C with continous stirring, using Clarke-type electrode. Additions: oligomycin, 5 μg/ml (olig); 10 μM aurovertin B (auro); 0.1 mM DNP; 4 μM rotenone (rot)

oligomycin-sensitive ROS production (not shown), so non-mitochondrial source(s) of ROS triggered by late apoptotic events downstream of mitochondria may be suggested. As to effect of oligomycin, it might be a consequence of inhibition of some events at the mitochondrial level, which result in an ROS over-production outside mitochondria.

PTP is involved in the oligomycin-sensitive, TNF-induced apoptosis

The effect of oligomycin was not related to decrease in the ATP/ADP ratio due to cessation of oxidative phosphorylation as far as inhibitors of respiration and uncouplers did not affect the TNF-induced apoptosis (Figure 2c). Possible hyperpolarization of mitochondrial membrane caused by inhibition of H^+-ATP-synthase also was not responsible for inhibition of apoptosis with oligomycin. In fact, oligomycin suppressed the TNF-induced apoptosis in combination with uncoupler which is known to dissipates proton motive force (Figure 2c) or with respiratory inhibitors arresting its generation (not shown). We may also assume that inhibition of the mitochondrial H^+-ATP-synthase activity is not critical for the effect of oligomycin since another inhibitor of this enzyme, aurovertin B, mimicked oligomycin in the effect on respiration (Figure 1) but did not affect the TNF-induced apoptosis (Figure 2c).

It was earlier shown in our laboratory (Novgorodov *et al*., 1989) that oligomycin strongly inhibited the PTP opening in isolated mitochondria. We have addressed the role of PTP in the Sts- and TNF-induced apoptoses using Cyclosporin A (CsA), the selective inhibitor of the PTP. It was found that CsA in combination with trifluoropyrasine (TFP), the inhibitor of phospholipase A_2, inhibited the TNF-induced release of cytochrome *c* and apoptosis (Figures 2c and 3c). Neither CsA nor TFP were effective alone (this is in line with the temporary inhibition of PTP by CsA in isolated mitochondria and prolongation of the CsA effect by TFP (Broekemeier and Pfeiffer, 1995)). MeVal-CsA, a CsA analogue effective in PTP inhibition but ineffective in inhibition of calcineurin, also suppressed TNF-induced apoptosis (not shown) so a role of calcineurin, the major non-mitochondrial CsA target, could be excluded.

One more piece of evidence for the PTP involvement in effects of TNF and oligomycin was obtained when electron microscopy of HeLa cells was done. It was found that at late stage of the TNF apoptosis all mitochondria are converted to small spherical bodies with very electron-dense matrix (the ultracondensed state, see also Zhuang *et al*., 1998). At earlier apoptotic stages, mitochondrial swelling was observed accompanied by an increase in the size of the organelles, the matrix space being electron-transparent. Just these changes could be expected, if the PTP is opened. A 5 h treatment with TNF and emetine proved to be the most convenient to observe the effect of oligomycin since at this stage oligomycin, in fact, completely inhibited apoptosis (see above, Figure 2d). At such a stage, in some cells mitochondria were swollen (c.f. Figure 5a,b) whereas in the others they were already ultracondensed (Figure 5c). Oligomycin (Figure 5d) and Bcl-2 (not shown) suppressed these effects.

The Sts-induced apoptosis was not inhibited by either oligomycin or CsA and TFP (Figure 2b). Recently, however, the PTP-dependent mechanism was also suggested for Sts-induced killing of HeLa cells (Tafani *et al*., 2001). In this work, Sts added to a serum-free medium at very low concentration (20 nM) caused the cell death accompanied by a damage to the plasma membrane. Cells were protected by bongkrecate, an inhibitor of the ANT and the PTP. We have reproduced the conditions described and observed the

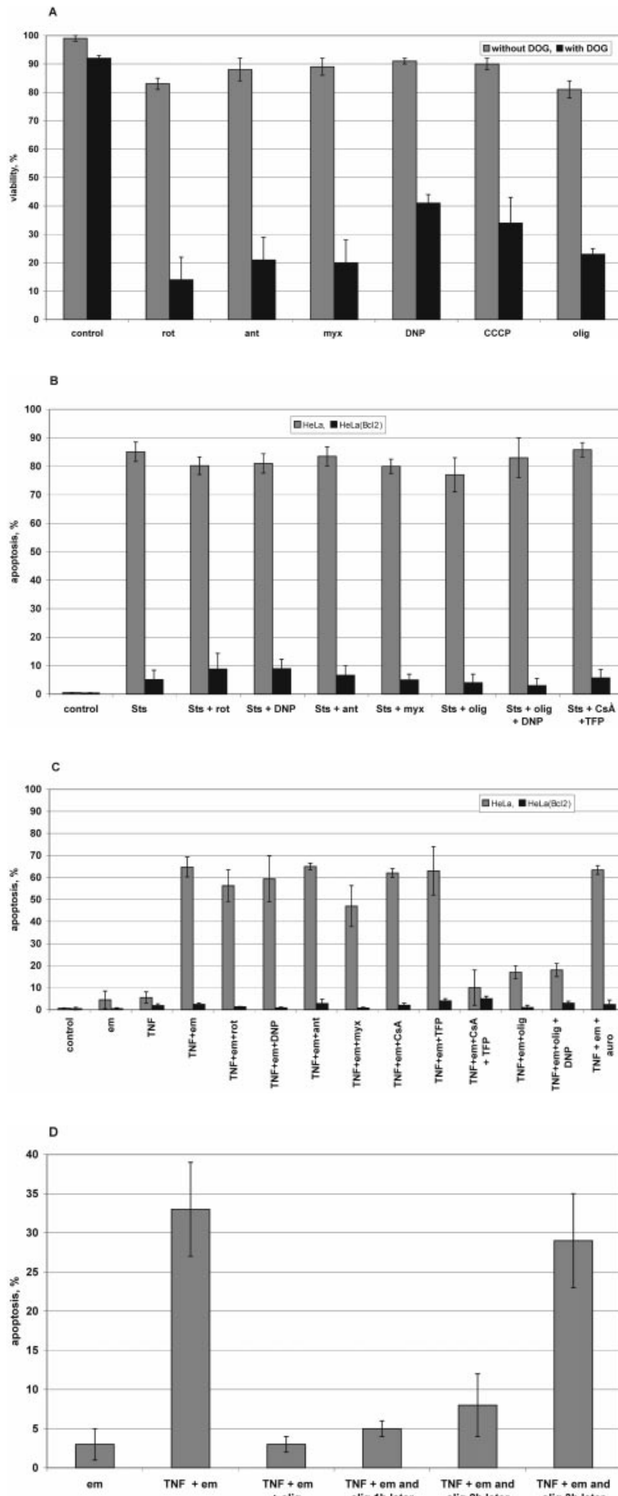


Figure 2 Apoptotic cell death induced by Sts or TNF: effects of mitochondrial inhibitors. The cell viability (**a**) was measured using MTT-test (see 'Materials and methods'). The percentage of apoptotic cells (**b**–**d**) that had condensed and fragmented nuclei was determined by staining with Hoechst 33342 (1 μg/ml) after fixation with 4% paraformaldehyde. 500 nuclei were counted in every sample. (**a**) Viability was measured after 24 h incubation in the presence (dark columns) or in the absence (white columns) of 5 mM DOG with or without the following mitochondrial inhibitors: 2 μM rotenone (rot); 2 μM antimycin A (ant); 1 μM myxothiazol (myx); 0.1 mM DNP; 5 μM CCCP; oligomycin, 5 μg/ml (olig). Results of five experiments. (**b**) Apoptosis induced by an 8 h treatment with Sts (1 μg/ml) in HeLa (white columns) and HeLa(Bcl-2) (dark columns). Additions: 20 μM TFP; 5 μM CsA; for the others, see (**a**). Results of five experiments. (**c**) Apoptosis induced by an 8 h treatment with TNF (5 ng/ml) in HeLa (white columns) and HeLa(Bcl-2) (dark columns). Additions: emetine, 1 μg/ml (em); 10 μM aurovertin B (auro); for the other additions, see (**a** and **b**). Results of five experiments. (**d**) Effect of oligomycin added together with, or some time after, TNF and emetine. Oligomycin was added immediately, or in 1, 2, 3 h after TNF and emetine. The full time of treatment with TNF and emetine was 5 h. For additions, see **a**–**c**. Results of three experiments

typical apoptosis (without disruption of the plasma membrane) which was insensitive to CsA in combination with TFP (or CsA alone) and to oligomycin (data not shown). These data confirm the conclusion that in HeLa cells the mechanisms of cytochrome *c* release induced by TNF and Sts (even if the later included some form of the PTP) are different.

Discussion

The data obtained allow us to conclude that in HeLa cells neither Sts- nor TNF-induced apoptoses, as well as their prevention by Bcl-2, require mitochondrial respiration or changes in the mitochondrial membrane potential. Some changes of these parameters during apoptosis observed by others (see, e.g. Vander Heiden *et al*., 1997) may be regarded as epiphenomena accompanying, but not required for, the programmed death of HeLa cells, initiated by Sts or TNF.

The same conclusion can be made concerning ATP hydrolysis due to reversal of H^+-ATP-synthase. Aurovertin B at concentrations arresting this enzyme in HeLa cells (see inhibition of the cell respiration, Figure 1, trace 2) was found to be without effect on apoptosis (Figure 2c). On the other hand, oligomycin, another H^+-ATP-synthase inhibitor, was found to suppress the TNF- (but not Sts-) induced apoptosis and related events (Figures 2–5).

The anti-apoptotic action of oligomycin was described earlier (see Introduction) but the mechanism of the effect remained unclear. Yaguzhinsky and his colleagues showed that oligomycin inhibited the PTP opening in isolated mitochondria and the effect was attributed to accumulation of ADP (a well known inhibitor of the PTP) in the matrix (Novgorodov *et al*., 1989). The same effect probably underlies inhibition by oligomycin of cytochrome *c* release from isolated mitochondria caused by Bax (Narita *et al*., 1998). On the other hand, the effect described in our paper was not related to accumulation of ADP because (i) oligomycin inhibited the TNF-induced apoptosis in the presence of uncoupler or inhibitors of respiration, when phosphorylation of ADP was already blocked, and (ii) inhibition of ATP synthesis with aurovertin B did not affect apoptosis (Figure 2c). Apparently, the mechanism of the PTP opening, which seems to be induced by activated Bid in the case of TNF-induced

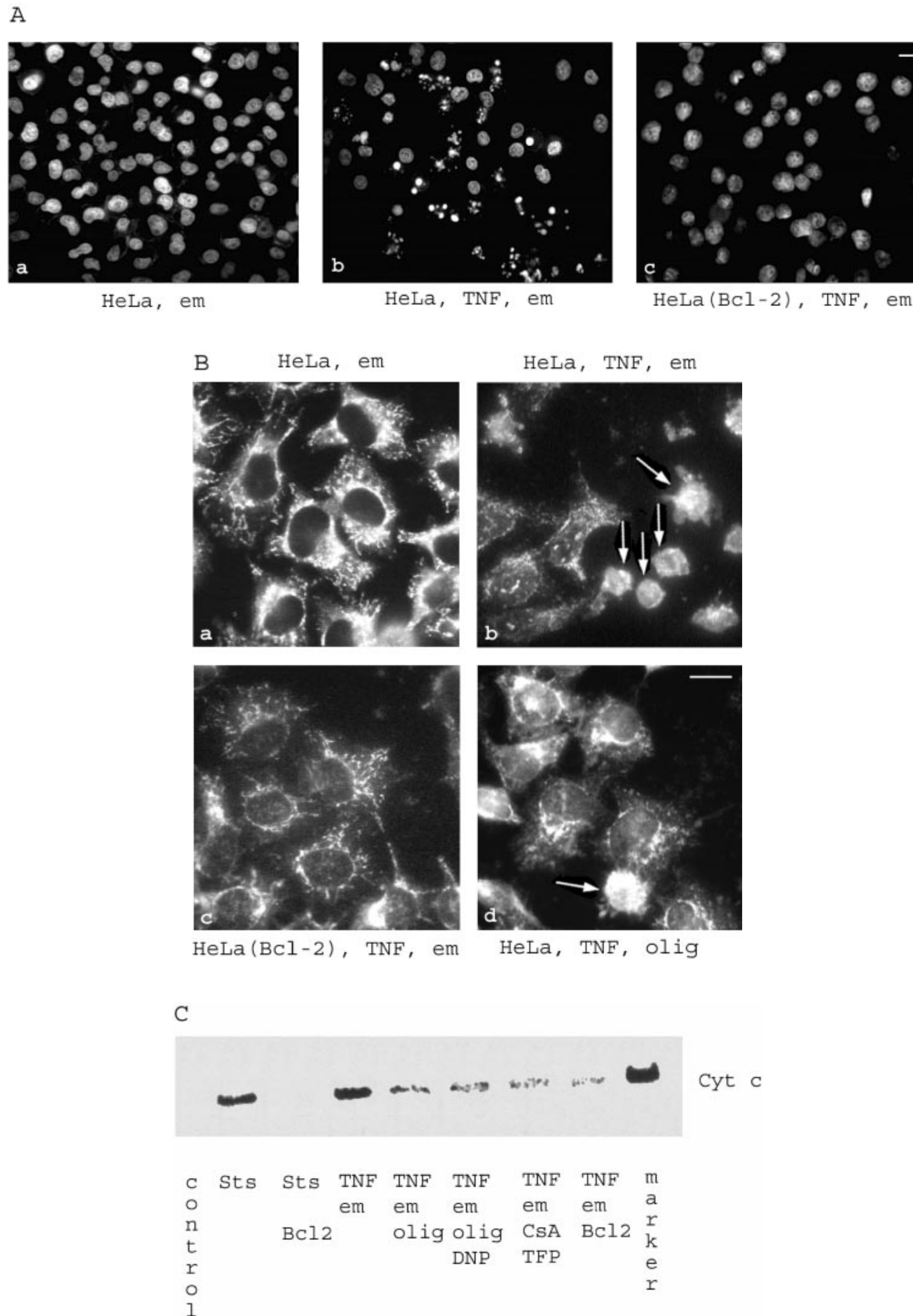
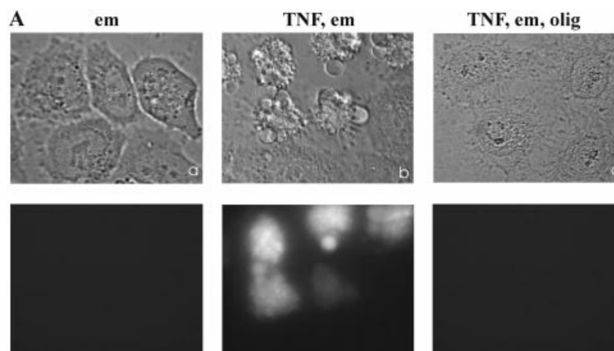


Figure 3 Chromatin condensation and fragmentation (**a**), and release of cytochrome *c* from mitochondria into cytosol (**b**,**c**) during apoptosis in HeLa and HeLa(Bcl-2). (**a**) Cells were treated with emetine, 1 μg/ml (em) and TNF, 5 ng/ml, for 8 h, fixed and stained with Hoechst 33342. Bar, 30 μm. (**b**) Cells were treated with emetine, TNF (**b**–**d**) and oligomycin, 5 μg/ml (**d**), for 8 h. Immunostaining of cytochrome *c* with 7H8.2C12 anti-cytochrome *c* antibodies (1 μg/ml). Arrows indicate apoptotic cells. Bar, 30 μm. The percentage of the cells with cytochrome *c* released into cytosol was the following: (**a**) <5%; (**b**) 63%; (**c**) <5%; (**d**) 18%. A total of 300 cells were analysed in each sample. (**c**) Immunoblots of cytosolic extracts with 7H8.2C12 anti-cytochrome *c* antibodies. Horse cytochrome *c* was used as a marker (Cyt *c*). Cells were treated for 8 h with Sts, 1 μg/ml, emetine, 1 μg/ml (em) and TNF, 5 ng/ml. Concentrations of additions as in Figure 2

apoptosis, is different from that triggered in isolated mitochondria by Ca^{2+} or uncouplers and inhibited by oligomycin in an ADP-dependent manner. An alternative possibility is that the PTP opening is necessary but not sufficient for cytochrome *c* release induced by TNF and that some other event(s) (structural changes in mitochondrial cristae (Scorrano *et al*., 2002), oxidation of cardiolipin (Ott *et al*., 2002), etc.) are



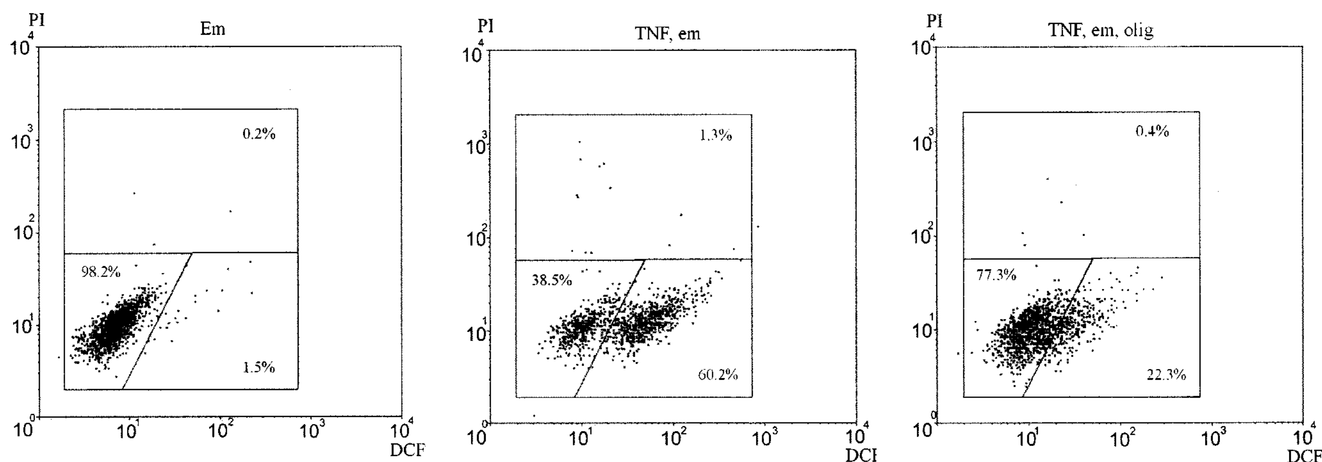


Figure 4 The effect of oligomycin on the TNF-induced overproduction of ROS. Cells were treated with emetine, 1 μg/ml (em), TNF, 5 ng/ml, and oligomycin, 5 μg/ml (olig) for 5 h; loaded with DCFDA (5 μM, 15 min) and analysed with microscopy and FACS. (**a**) Vital images: phase-contrast and fluorescence (upper and lower rows, respectively) of the same fields. (**b**) Flow-cytometric analysis. Dichlorofluorescin (DCF) and propidium iodide (PI) fluorescence are presented. The cells are divided in three populations: (i) PI-positive; (ii) PI-negative, DCF-low, and (iii) PI-negative, DCF-high. The percentage of cells in these populations in a typical experiment (of three experiments) is presented

required. These events might depend on the native structure of the membranous (F_o) component of the H^+- ATP-synthase, which is affected by oligomycin, but not of its water-exposed (F_1) component affected by aurovertin B. At present, such a hypothesis seems to be the simplest one. On the other hand, it is impossible to exclude that there is some other, still unknown oligomycin target responsible for the observed anti-apoptotic effect of the antibiotic. This is hardly Bax oligomerization (Ikemoto *et al*., 2000) since, if it were the case, the Sts-induced apoptosis would also be oligomycin-sensitive.

The mechanism of cytochrome *c* release from mitochondria related to opening of the PTP was suggested for the TNF-induced apoptosis in hepatocytes (Bradham *et al*., 1998) and L929 fibrosarcoma cells (Tafani *et al*., 2000). Our experiments, where CsA was used as an inhibitor of the pore, supported the key role of this phenomenon in apoptosis induced by TNF, but not by Sts, in HeLa cell (Figure 2). The experiments with inhibitors of respiration and with uncouplers exclude some mechanisms of the pore induction, namely, excessive Ca^{2+} accumulation in the matrix, decrease of the membrane potential, oxidation of the matrix components and ROS produced by the respiratory chain in energized mitochondria, which were impossible when the membrane potential was abolished by an uncoupler or respiration was arrested by a respiratory chain inhibitor.

It is noteworthy that recently Peachman *et al*. (2001) described an opposite (pro-apoptotic) effect of oligomycin on eosinophils. These cells are unique in that the major function of their mitochondria seems to consist in promoting apoptosis rather than in energy transduction. The number of mitochondria is small, their respiration is slow and cyanide-resistant, mitochondrial membrane potential is generated at the expense of glycolytic ATP, respiration being ineffective. Normally eosinophils die *in vivo* and *in vitro* by apoptosis. Oligomycin and, to a lesser degree, uncouplers were found to stimulate cytochrome *c* release and apoptosis. The authors assumed that the loss of mitochondrial membrane potential alone is not sufficient to initiate

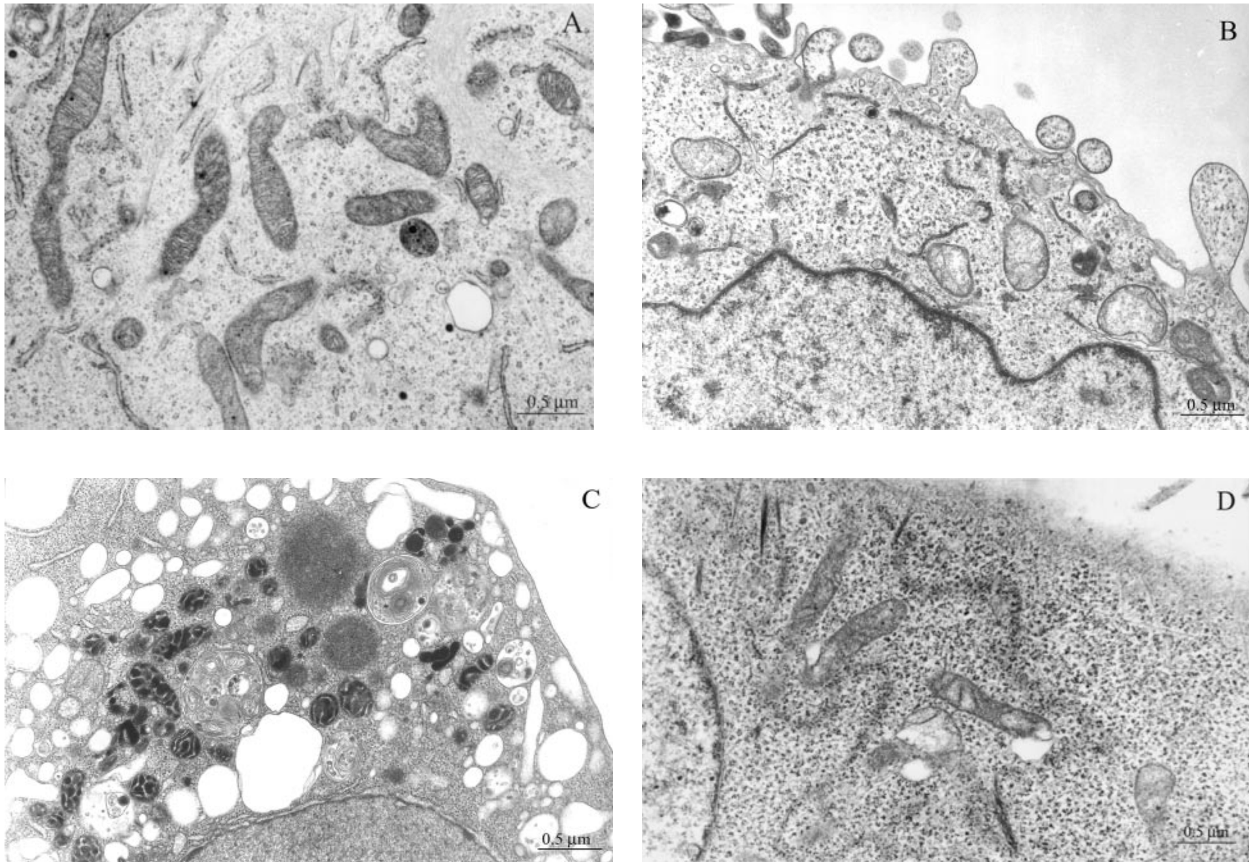


Figure 5 Electron microscopy of HeLa cells treated for 5 h with emetine (**a**), emetine and TNF (**b**,**c**) and emetine, TNF and oligomycin (**d**). For concentrations of additions, see Figure 2. In **b** and **c**, cells at an early and a late stage of apoptosis are shown, respectively

apoptosis in eosinophils since uncouplers also reducing membrane potential proved to be less efficient than oligomycin as proapoptotic agents. However, it is not excluded that in the uncoupler-treated cells low membrane potential was still maintained but could not be measured with the probe used by Peachman *et al*. (2001). As to oligomycin, it completely abolished membrane potential. Such relationships are not surprising if we take into account that oligomycin prevents the membrane potential formation whereas uncouplers lower already formed membrane potential. Thus, the effects observed might be explained by de-energization of mitochondria. This is in contrast to our observation that oligomycin proved to be anti-apoptotic and its action was not reproduced by uncouplers.

Materials and methods

Human carcinoma cells HeLa were grown in DMEM medium supplemented with high glucose (25 mM), gentamycin sulphate (0.08 mg/ml) and 10% FCS (Gibco) (the growth medium) at 37°C and 5% CO_2. The HeLa and HeLa(Bcl-2) cell lines were provided by Dr GA Belov (MP Chumakov Institute of Poliomyelitis and Viral Encephalitides, Moscow, Russia). Human *bcl-2* gene was introduced into HeLa cells using pLPC-bcl-2 vector for transfection. Control clone was similarly prepared using an empty vector. Expression of Bcl-2 protein in transfected cells was confirmed by Western blot analysis using anti-human Bcl-2 monoclonal antibody.

Cell viability was determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reductase activity (Liu *et al*., 1997). Briefly, cells were grown and treated in 96-well plates then MTT (0.25 mg/ml final concentration) was added and cultivation was continued for 3 h. After removal of the medium pellets were dissolved by DMSO and optical density was measured at 570 nm using microplate reader. Six wells were analysed and the mean value was calculated for every point. Apoptosis was measured by determining the percentage of cells that had condensed and fragmented nuclei by staining with fluorescent dye Hoechst 33342 (1 μg/ml) after fixation with 4% paraformaldehyde. Five hundred nuclei were counted in every sample. Necrotic cells were detected by failure of Trypan blue exclusion.

Respiration was measured with a Clarke-type electrode at 37°C in Hanks Balance Salt medium with 10 mM glutamine and 30 mM HEPES, pH 7.3. $5\times10^6-10^7$ cells were suspended in 1 ml of the medium placed into the closed chamber and recording was started immediately.

For immunostaining, cells were grown on glass cover slips and fixed by a 15 min incubation with ice-cold methanol at -20°C. Probes were incubated with monoclonal anti-

cytochrome *c* antibodies (1 μg/ml, 7H8.2C12, PharMingen) and were stained with FITC-tagged secondary anti-mouse IgG antibodies. Cells in Vectashield medium were analysed under a fluorescence microscope (Axiophot, Zeiss, Germany) equipped with an MTI CCD-camera (Scion Image software was employed).

Cells were fractionated after disruption with the conical teflon pestle in Eppendorf tube for 20 s in the medium containing 125 mM KCl, 75 mM sucrose, 20 mM HEPES-NaOH, pH 7.4, 1 mM EDTA, 10 mM dithiothreitol, 10 μM phenylmethylsulphonylfluoride, 10 μM leupeptin, 10 μM aprotinin at 4°C. The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant was centrifuged at 12 000 *g* for 30 min to prepare the cytosolic fraction. The samples were normalized for protein content (100 μg/lane) by the Bio-Rad protein assay, run on 15% SDS–PAGE and blotted onto nitrocellulose membrane (Amersham). Membranes were blocked with 5% (wt/vol) nonfat milk in 100 mM Tris-HCl (pH 7.5), 0.9% NaCl, 0.1% (v/v) Tween 20, subsequently incubated with 1 μg/ml of the 7H8.2C12 antibodies to cytochrome *c* (PharMingen) and stained with anti-mouse IgG conjugated with horseradish peroxidase (Calbiochem).

For detection of ROS production, cells ($10^6 - 5 \times 10^6$ cells/ml) were loaded with DCFDA (5 μM, 15 min at 37^0C), washed, suspended in the growth medium and analysed under the fluorescence microscope or using flow-cytometry (FACStar, Beckton-Dickinson). In the latter case, the medium was supplemented with propidium iodide (10 μg/ml, 5 min). In total 10 000 events were recorded in each analysis.

For electron microscopy, the cells were fixed with 2.5% glutaraldehyde in phosphate buffer, post-fixed with osmium tetroxide, dehydrated in ethanol solutions of increasing concentrations, and embedded in Epon-812. Ultrathin serial sections were prepared using LKB-III ultratome. The section were counterstained with lead citrate, and examined with Hitachi HU-11B electron microscope at accelerating voltage 75 kV.

Human recombinant TNF was a kind gift from Dr V Korobko (Institute of Bioorganic Chemistry, Moscow, Russia). DCFDA and Mitotracker Red were from Molecular Probes. Other reagents were from Sigma.

Abbreviations

ANT, adenine nucleotide translocator; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CsA, Cyclosporin A; DCF, dichlorofluorescine; DCFDA, dichlorofluorescine diacetate; DMEM, Dulbecco's modified Eagle's medium; DNP, 2,4-*p*-dinitrophenol; DOG, 2-deoxiglucose; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PTP, permeability transition pore; Sts, staurosporine; TFP, trifluoropyrasine; TNF, tumor necrosis factor α.

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