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# Glutamine delays spontaneous apoptosis in neutrophils

**Tania C. Pithon-Curi,[1] Robert Ivan Schumacher,[2] Jofre J. S. Freitas,[3] Cláudia Lagranha,[3] Philip Newsholme,[4] Adrianne C. Palanch,[1] Sonia Q. Doi,[5] and Rui Curi[3]**

[1]*Methodist University of Piracicaba, FACEF, and Camilo Castelo Branco University, São Paulo;* [2]*Department of Biochemistry and* [3]*Department of Physiology and Biophysics, University of São Paulo, São Paulo, SP, Brazil;* [4]*Department of Biochemistry, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland; and* [5]*Uniformed Services University, Bethesda, Maryland 20814*

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**Pithon-Curi, Tania C., Robert Ivan Schumacher, Jofre J. S. Freitas, Cláudia Lagranha, Philip Newsholme, Adrianne C. Palanch, Sonia Q. Doi, and Rui Curi.** Glutamine delays spontaneous apoptosis in neutrophils. *Am J Physiol Cell Physiol* 284: C1355–C1361, 2003. First published January 15, 2003; 10.1152/ajpcell.00224.2002.—Nuclear, mitochondrial, and plasma membrane events associated with apoptosis were investigated in rat neutrophils cultivated for 3, 24, and 48 h in the absence or presence of glutamine (0.5, 1.0, and 2.0 mM). Condensation of chromatin was reduced after 24 or 48 h of culture in the presence of glutamine compared with its absence as assessed by Hoechst 33342 staining. The level of *Escherichia coli* phagocytosis in the presence of glutamine was markedly increased compared with the level achieved by cells cultured in the absence of glutamine. Annexin V binding to externalized phosphatidylserine was reduced in the presence of glutamine. Sensitive fluorochrome rhodamine 123, as determined by fluorescence-activated cell sorting and confocal microscopy, was used to monitor loss of the mitochondrial transmembrane potential. In the absence of glutamine, neutrophils exhibited a marked reduction in the uptake of rhodamine 123. In the presence of 1.0 or 2.0 mM glutamine, the uptake of rhodamine was 20 or 38% higher, respectively. Similar effect was found in human neutrophils by measuring DNA fragmentation and mitochondrial transmembrane potential. Therefore, glutamine protects from events associated with triggering and executing apoptosis in both rat and human neutrophils.

mitochondria; neutrophil

NEUTROPHILS CONSTITUTE 60% of the circulating leukocytes. They act as first-line-of-defense cells in the plasma and undergo phagocytosis either directly or in cooperation with antigen-specific defenses. Neutrophils are terminally differentiated end cells and are produced in the bone marrow from myeloid stem cells. These cells survive for a short time (8–20 h) in the blood and undergo apoptosis, presenting morphological changes such as diminution in cell volume, nuclear condensation, and cytoplasmic vacuolation (6, 16). There is also internucleosomal cleavage of DNA, resulting in hypodiploid nuclei (2). It is noteworthy that the half-life of the neutrophils increases severalfold once they enter infected or inflamed tissues (1). Several inflammatory cytokines including granulocyte/macrophage colony-stimulating factor, IL-2, TNF-α, and IL-15 have been reported to prolong neutrophil survival (18). The delay of neutrophil apoptosis induced by cytokines is postulated to occur by induction of transiently expressed proteins that regulate apoptosis (3) or by activation of preexisting proteins (e.g., via phosphorylation). This issue, however, still remains controversial, because apoptosis is a very complex phenomenon involving a regulated series of events, in part controlled by extracellular stimuli including cytokines and perhaps nutrient availability (8).

The main substrate for ATP production in neutrophils has been reported to be glucose (5). Recent studies, however, have shown that rat neutrophils utilize glutamine at higher rates than glucose (5). Glutamine is also utilized at high rates by lymphocytes and macrophages (4). Glutamine has been shown to be important for the production of cytokines such as IL-1β, IL-6, TNF-α, and IL-8 from immunostimulated macrophages and monocytes (19, 20, 24). In neutrophils from burn and postoperative patients, glutamine augments the in vitro bacterial killing activity (22), and it is also important for optimization of the rate of production of reactive oxygen species (10). The pathway of glutamine metabolism in neutrophils is postulated to be similar to that reported for lymphocytes and macrophages, where glutamine is only partially oxidized. However, glutamine metabolism is important for NADPH and ATP production and the biosynthesis of proteins (e.g., cytokines) and lipids (e.g., triacylglycerol) (19, 20).

Nunn et al. (21) determined the endogenous concentration of various metabolites in human neutrophils undergoing apoptosis, including lactate and some amino acids. The endogenous concentration of lactate and glutamine was reduced by 45%, whereas that of arginine, glycine, alanine, aspartate, and glutamate was not modified. Thus glutamine utilization may have

Address for reprint requests and other correspondence: T. C. Pithon-Curi, Departamento de Fisiologia e Biofísica, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes 1524, 05508-900 Butantan, São Paulo, SP, Brazil (E-mail: tcuri@fisio.icb.usp.br).

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been increased in the apoptotic neutrophil. Because of the high rates of glutamine utilization by neutrophils, we hypothesized that this amino acid may contribute to mitochondrial functionality, ATP generation, and protection from apoptosis. Thus the effect of glutamine on parameters of rat and human neutrophil apoptosis was assessed, including chromatin condensation, DNA fragmentation, phosphatidylserine externalization, and changes in mitochondrial transmembrane potential (MTP), utilizing fluorescence-activated cell sorter (FACS) analysis and confocal microscopy. The effect of glutamine on neutrophil phagocytosis was also determined because a loss of mitochondrial function, associated with early events of apoptosis, would reduce phagocytic capacity (30).

## MATERIALS AND METHODS

*Animals.* Male Wistar rats weighing 180 g (~2 mo of age) were obtained from the Institute of Biomedical Sciences (University of São Paulo). The rats were maintained at 23°C under a 12:12-h light-dark cycle. The Animal Care Committee of the Institute of Biomedical Sciences approved the experimental procedure of this study.

*Reagents.* The following reagents were obtained: RPMI 1640, phosphate-buffered saline, penicillin-streptomycin, and fetal calf serum (GIBCO-BRL, Gaithersburg, MD); ethidium bromide (Bio-Rad, Hercules, CA); Light Antifade kit, annexin V-fluorescein isothiocyanate (FITC), Hoechst 33342, Vybrant phagocytosis assay kit (Molecular Probes, Eugene, OR); and L-glutamine, type II glycogen from oyster, 6-diazo-5-oxo-L-norleucine (DON), cytochrome *c*, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), rhodamine 123, oligomycin, glucose, citrate, Triton X-100, trypan blue, and propidium iodide (PI) (Sigma, St. Louis, MO).

*Preparation of rat neutrophils.* The cells were obtained from rats killed by decapitation without anesthesia. Neutrophils were obtained by lavage of the peritoneal cavity with 40 ml of sterile PBS, 4 h after the intraperitoneal injection of 20 ml of sterile oyster glycogen solution (1% in PBS). The cell suspension was centrifuged at 4°C (850 *g* during 8 min) three times in PBS.

*Preparation of human neutrophils.* Human neutrophils were isolated from peripheral blood of healthy adult men volunteers. Blood (10 ml) was diluted with an equal volume of PBS at pH 7.4 containing 100 mM $CaCl_2$-50 mM $MgCl_2$ and carefully layered on 10 ml of a commercial gradient of Lymphoprep (density = 1.077). The tube was centrifuged at 1,200 rpm at 4°C for 30 min. The supernatant, rich in mononuclear cells, was discarded. The pellet was submitted to hypotonic treatment with 10 ml of solution containing 150 mM $NH_4Cl$, 10 mM $NaHCO_3$, and 0.1 mM EDTA to promote lysis of contaminated erythrocytes. The tube was homogenized and maintained for 10 min at ice to allow erythrocyte lysis. The tube was then centrifuged at 1,200 rpm at 4°C for 10 min. This procedure was repeated twice.

Neutrophils obtained from rats and humans were counted in a Neubauer chamber under an optical microscope. The number of viable cells, always >95% neutrophils, was determined by trypan blue exclusion.

*Culture of neutrophils.* The cells ($1.0 \times 10^6$ cells/ml) were seeded in RPMI 1640 medium containing 11.1 mM glucose but no glutamine. The medium was supplemented with 10% (vol/vol) fetal calf serum, and the cells were cultivated in the absence and presence of glutamine (0.5, 1, and 2 mM) and penicillin-streptomycin (20 μg/ml) as previously described (5). The cells were cultured at 37°C in 95% air-5% $CO_2$ for up to 48 h depending on the measurement performed.

*Chromatin condensation assay.* Neutrophils were cultured at 37°C in 95% air-5% $CO_2$ for 3, 24, and 48 h in the absence and presence of glutamine (2 mM). At the end of each period, cells were resuspended in 20 μl of 0.9% NaCl solution containing 0.01 mg/ml Hoechst 33342 and then incubated for 15 min at room temperature. The cells were mounted on glass slides using glycerol from the Light Antifade kit. Slides were observed by fluorescence microscopy (Axiovert 100 M; Zeiss, Zeppelinstrasse, Germany) under UV light (365 nm/380 nm). The images were analyzed using the KS 300 Image System 3.0 software (Zeiss).

*DNA fragmentation.* PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per four to five base pairs of DNA. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold, the fluorescence excitation maximum is shifted ~30–40 nm to the red, and the fluorescence emission maximum is shifted ~15 nm to the blue. PI is membrane impermeant and generally excluded from viable cells. PI is commonly used for identifying dead cells in a population and as a counterstain in multicolor fluorescent techniques.

Neutrophils ($1 \times 10^6$) were incubated for 24 h in the dark in a solution containing 0.1% citrate, 0.1% Triton X-100, and 50 μg/ml PI. Cells with PI fluorescence were then evaluated by flow cytometric analysis using the Cell Quest software. Results are presented as histograms of PI fluorescence (%).

*Measurement of phosphatidylserine externalization by using annexin V binding assay.* Neutrophils were cultured for 3 h in the absence or presence of 1 or 2 mM glutamine. After treatments, $2 \times 10^5$ cells were harvested, washed in ice-cold PBS, and resuspended in 200 μl of annexin binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$). The cells were stained with annexin V-FITC for 10 min on ice in the dark, according to the manufacturer's instructions, and analyzed in a confocal microscope (Axiovert 100 M). The images were analyzed by using the LSM-510 software (Zeiss). A similar procedure was used by Vermes et al. (28).

*Mitochondrial transmembrane potential.* Cells were cultivated ($5 \times 10^6$ cells/ml) in the absence and presence of glutamine at various concentrations (0.5, 1, and 2 mM) for 3 h. The following additions were also tested: 10 μM of the glutamine analog DON, 10 μM of CCCP (treatment for 30 min) as negative control (27), and 10 μM oligomycin (treatment for 30 min) as positive control. DON is known to be a potent catalytic inhibitor of the renal-type glutaminase activity (26). FACS and confocal microscopic analysis monitored changes in MTP. After the treatments, the cells were incubated for 30 min (FACS analysis) or 90 min (confocal microscopic analysis) in the presence of the fluorochrome rhodamine 123 (10 μM) in 1 ml of PBS. This dye is potential-sensitive and has been widely used as an indicator of the MTP (15). The results were compared between the different treatments and with cells immediately after being obtained.

*Phagocytosis.* The phagocytosis assay was carried out using the Vybrant phagocytosis assay kit (Molecular Probes). Neutrophils ($1 \times 10^6$ cells/ml) were mixed with $2 \times 10^5$ FITC-labeled *Escherichia coli* cells at 0°C. This mixture was incubated at 37°C in a horizontal shaker for 2 h. After two washes with PBS, the cells were mounted on glass slides using glycerol from the Light Antifade kit, according to the manufacturer's instructions. Slides were observed using fluorescence microscopy (Axiovert 100 M) under UV light (450 nm/520 nm).

*Determination of the mean gray values of relative fluorescence.* The images of Hoechst 33342 staining, phosphatidylserine externalization, MTP, and neutrophil phagocytosis were analyzed using the KS 300 Image System 3.0 software (Zeiss). The mean gray values were determined from relative fluorescence intensity of individual cells from three fields of slides prepared from three different experiments. The results are presented as mean gray values ± SD of at least 30 cells.

## RESULTS

*Evidence that glutamine delays the process of neutrophil apoptosis.* The level of Hoechst 33342 staining of rat neutrophils cultured in the absence or presence of 2 mM glutamine at 3, 24, and 48 h is clearly reduced in glutamine-incubated cells at the later time points (Fig. 1). The mean gray values shown in Fig. 1, *A–F*, were 103.8 ± 7.1, 103.5 ± 7.8, 86.9 ± 5.7, 188.7 ± 3.7, 76.3 ± 5.2, and 173.2 ± 5.8, respectively. Cells that have reached the terminal stage of apoptosis, in which chromatin condensation occurs, present an area clearly visible because of bright staining with Hoechst 33342 (Fig. 1, 24 and 48 h). This effect was more pronounced in rat neutrophils incubated in the absence of glutamine (Fig. 1, *D* and *F*). Glutamine either at 1 and 2 mM prevented phosphatidylserine externalization after 3 h of incubation as assessed by annexin V binding (Fig. 2). The mean gray values shown in Fig. 2, *A–C*, were 59.6 ± 11.8, 26.6 ± 8.8, and 0, respectively. A similar delayed effect of glutamine on apoptosis was observed in human neutrophils. In fact, glutamine

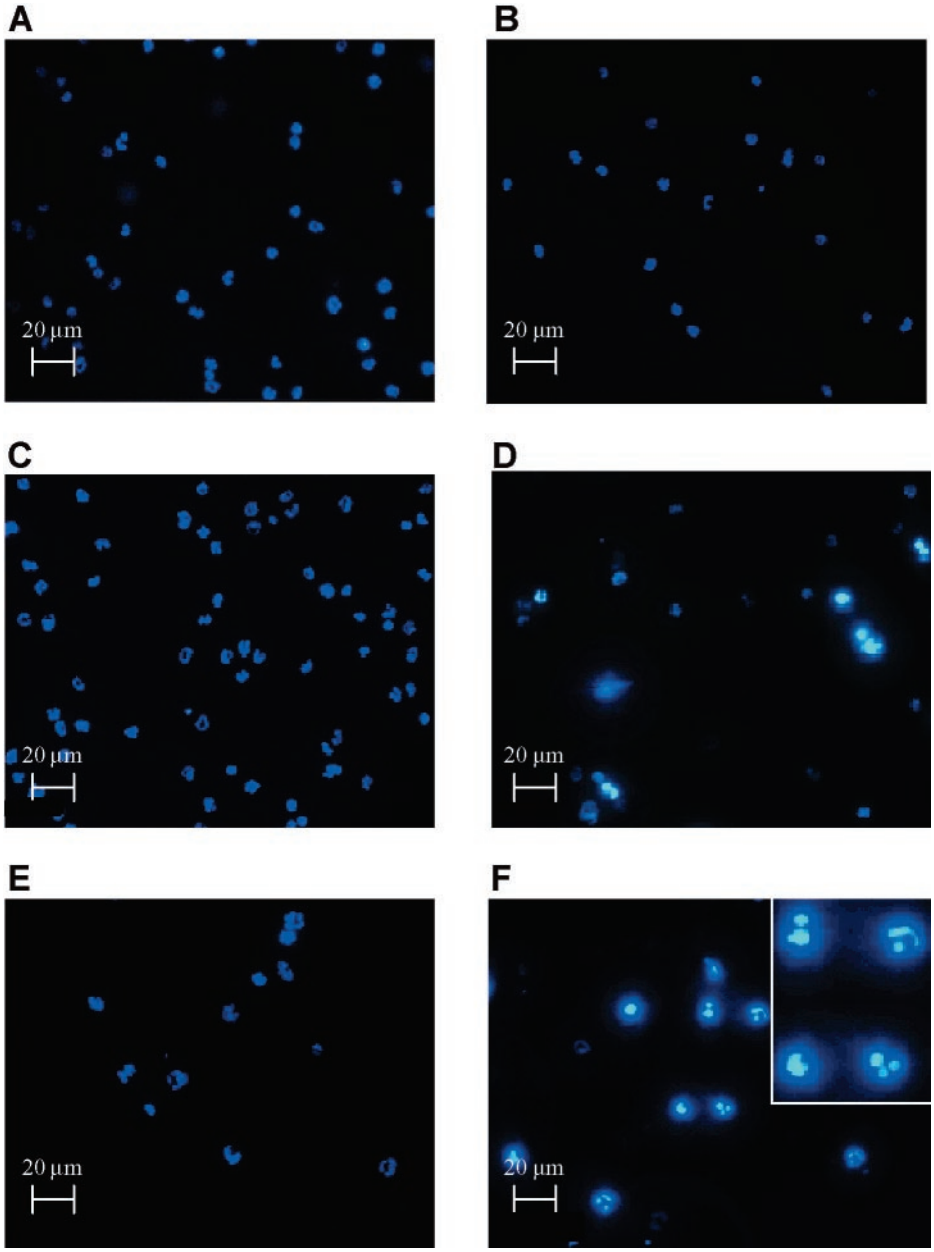


Fig. 1. Hoechst 33342 staining of neutrophils cultured in the absence (*B*, *D*, and *F*) or presence (*A*, *C*, and *E*) of glutamine. The neutrophils were incubated for 3 (*A* and *B*), 24 (*C* and *D*), or 48 h (*E* and *F*). The chromatin condensation and apoptotic bodies are shown in more detail in *F* (*inset*).

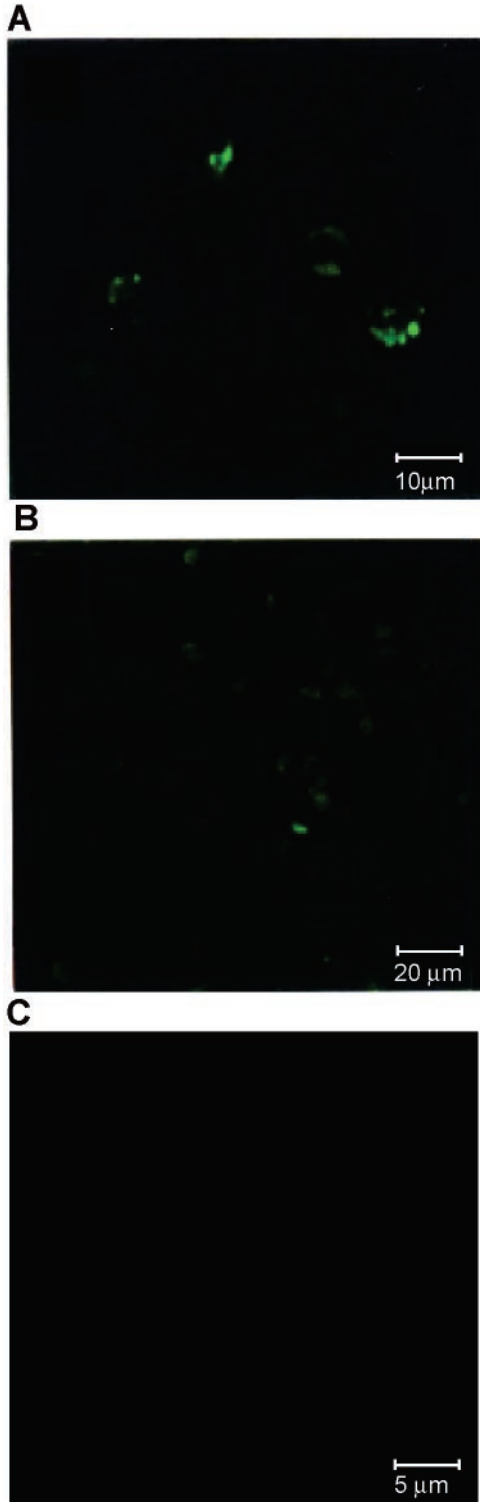


Fig. 2. Analysis by confocal microscopy of phosphatidylserine externalization in rat neutrophils incubated in the absence (*A*) and presence of 1 (*B*) or 2 mM (*C*) glutamine for 3 h. In *C*, the cells do not show phosphatidylserine externalization.

caused a dose-dependent decrease of DNA fragmentation in human neutrophils (Fig. 3).

*Glutamine-induced improvement in neutrophil mitochondrial function.* MTP of rat neutrophils was positively correlated with glutamine concentration as shown by confocal microscopy using rhodamine 123 (Fig. 4). The mean gray values shown in Fig. 4, *A–F*, were 48.2 ± 2.1, 64.4 ± 3.9, 10.8 ± 0.8, 50.3 ± 7.4, 30.6 ± 3.2, and 16.3 ± 1.6, respectively. Analysis of MTP was also achieved by FACS, using the same fluorochrome, in neutrophils cultivated for 3 h in the absence or presence of glutamine (0.5, 1, or 2 mM). The mean values of fluorescence intensities given by FACS were calculated and expressed as arbitrary units. The values were 453, 453, 542, and 626 for cells incubated in 0, 0.5, 1, or 2 mM glutamine, respectively. A similar effect of glutamine on MTP was observed in human neutrophils (Fig. 5).

The glutaminase inhibitor (DON) reduced the protective effect of glutamine on transmembrane potential (Fig. 4). We concluded that glutamine metabolism was

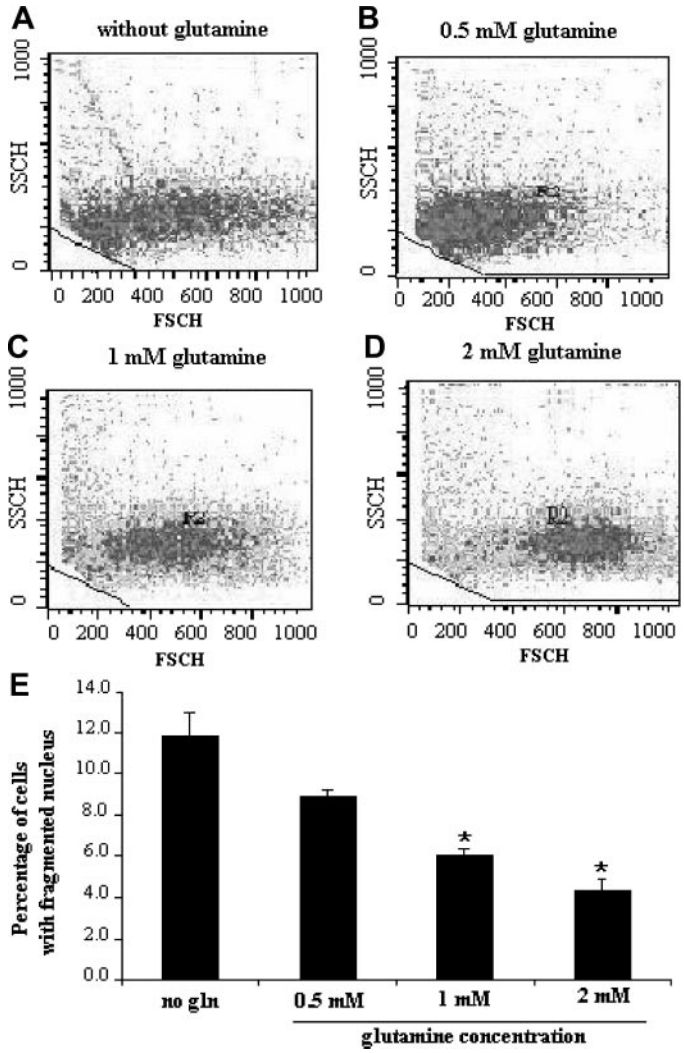


Fig. 3. Effect of glutamine on the percentage of fragmented nuclei of human neutrophils as monitored by flow cytometry using the sensitive fluorochrome propidium iodide. Neutrophils were cultured for 3 h in the absence (*A*) or presence of glutamine [0.5 (*B*), 1 (*C*), and 2 mM (*D*)]. *E*: values are presented as means ± SE of 3 separated experiments in duplicate and by analysis of 10,000 events. SSCH, side scatter channel; FSCH, forward scatter channel. *$P < 0.05$ due to the effect of glutamine.

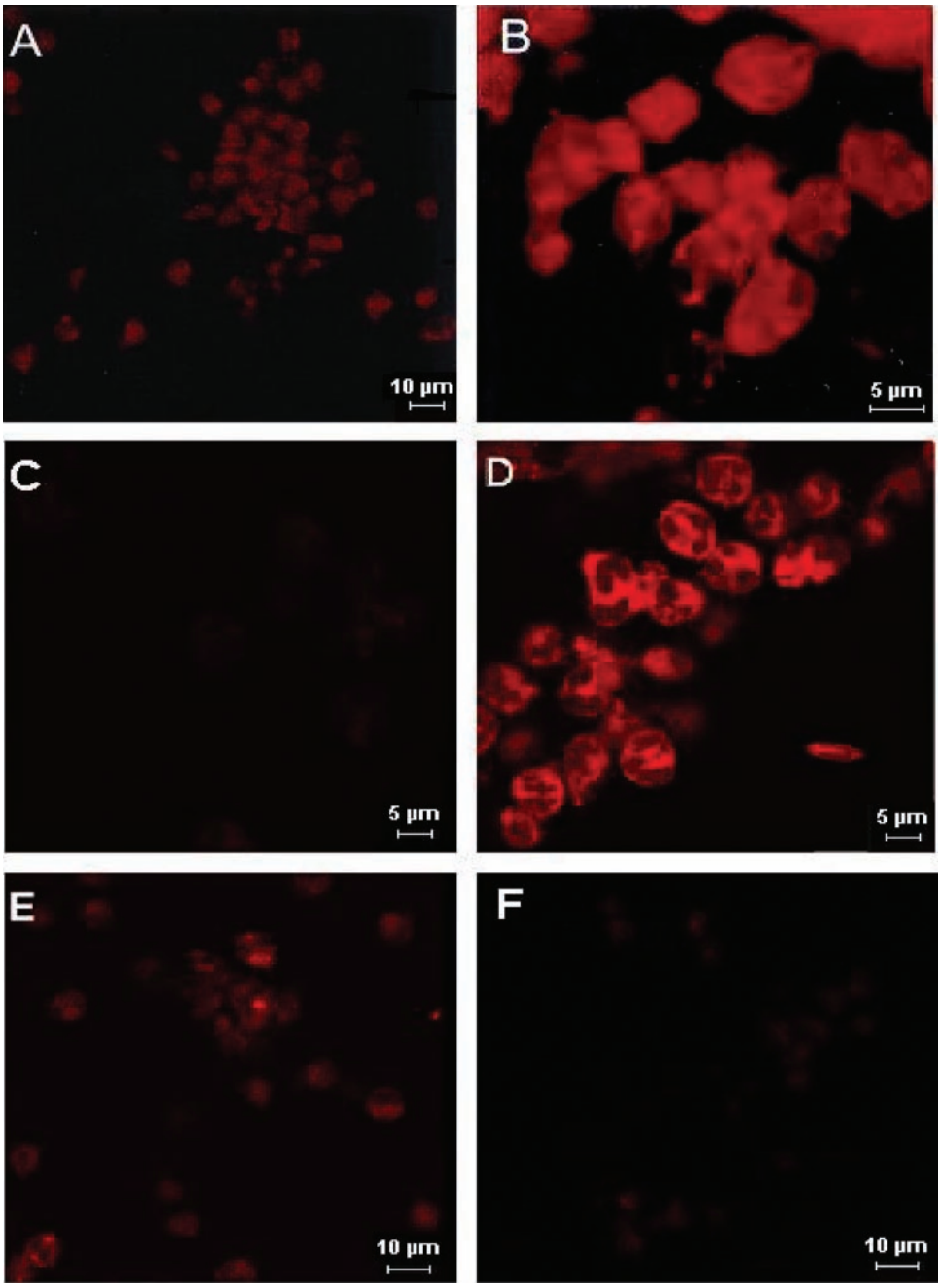


Fig. 4. Analysis by confocal microscopy of neutrophil mitochondrial transmembrane potential (MTP) monitored by assessing sensitive fluorochrome rhodamine 123 uptake 3 h after incubation. Fresh neutrophils were obtained by lavage of the peritoneal cavity (*A*). The cells were cultured with 2 mM glutamine and 10 μM oligomycin (to hyperpolarize the MTP, a positive control) (*B*) and in the absence (*C*) or presence of 2 mM glutamine (*D*). The cells were also cultured with 6-diazo-5-oxo-L-norleucine (DON; 10 μM), an inhibitor of phosphate-dependent glutaminase, plus glutamine (2 mM) (*E*) and in the absence of this amino acid but in the presence of DON (*F*).

important for maintenance of mitochondrial functionality.

Studying fluorescent *E. coli* neutrophil phagocytosis additionally tested the role of glutamine in the preservation of mitochondrial function. Rat neutrophils were cultured in the absence or presence of glutamine (2 mM). Glutamine-incubated neutrophils had a higher phagocytic capacity compared with cells cultured in the absence of this amino acid (Fig. 6). Opsonized zymosan (from *Saccharomyces cerevisiae*) phagocytosis was also carried out as described in our previous studies (17). Rat neutrophils cultured in the absence or presence of glutamine (2 mM) were tested for phagocytosis by counting the cells that had engulfed three or more particles of zymosan in a counting chamber. In the absence of glutamine there was a decrease of 25% in the neutrophils' phagocytic capacity (data not shown).

## DISCUSSION

Upon differentiation, neutrophils express decreased levels of antiapoptotic proteins and/or increased levels of proapoptotic proteins, which result in increased rates of apoptosis (7). As previously mentioned, neutrophils utilize glucose and glutamine at high rates (5), but the role of these metabolites for the process of apoptosis remains poorly understood. Two main pathways initiate apoptosis. The intrinsic pathway emerges

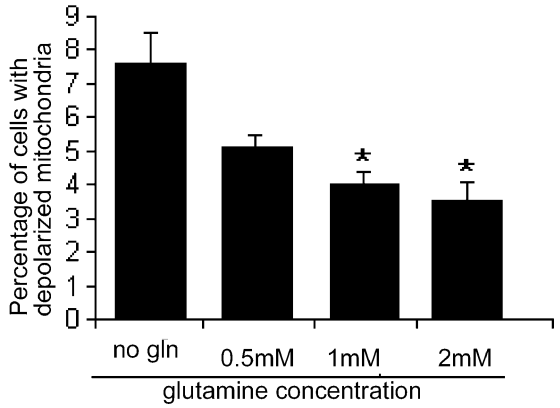


Fig. 5. Effect of glutamine on MTP of human neutrophils monitored by flow cytometry by assessing sensitive fluorochrome rhodamine 123 uptake after 3 h in cultured. Neutrophils were cultured in the absence or presence of glutamine (0.5, 1, and 2 mM). Values are presented as means ± SE of 3 separate experiments in duplicate. Gln, glutamine. *$P < 0.05$ due to the effect of glutamine addition.

from mitochondria, whereas the extrinsic pathway is activated by the activation of death receptors such as Fas receptors (29). Recently, Healy et al. (12) provided evidence that glucose can protect Fas-induced apoptosis in primary human neutrophils after 24 h in culture. These authors were unable to show a significant protective effect of glutamine. This study was mainly focused on the extrinsic pathway of cell death that does not necessarily involve mitochondrial events (29). In fact, Holler et al. (13) have shown that Fas kills activated primary T cells efficiently in the absence of active caspases, which results in necrotic morphological changes and only late mitochondrial damage with no release of cytochrome *c*. However, glutamine is mainly metabolized in mitochondria (4). In the present study, the role of glutamine on spontaneous neutrophils apoptosis was examined in a more detailed and systematic way. Glutamine presented a protective effect on neutrophils apoptosis that was initially observed as changes in mitochondrial function after only 3 h in culture. We have demonstrated that glutamine concentration was positively correlated with MTP and phagocytic capacity. We speculate that glutamine metabolism, via protective effects on mitochondrial integrity, delays spontaneous apoptosis in rat and human neutrophils.

Reduced MTP has been proposed to be necessary for the commitment of the cells to apoptosis (11). So far, changes in MTP can accelerate or slow the process of cell apoptosis. Glutamine did delay the mitochondrial disruption of both rat and human neutrophils.

Depletion of medium glutamine from cultured Chinese hamster ovary cells has been shown to enhance levels of apoptosis (25). One product of glutamine and glutamate metabolism is glutathione, which has been reported to stabilize neutrophil mitochondrial function and delay apoptosis (23). Therefore, the effect of glutamine to delay neutrophils apoptosis may have been mediated by the antioxidant effects of glutathione. In fact, reactive oxygen species are involved in neutrophil apoptosis stabilization of redox potential (11, 15). However, glutamine metabolism can result in formation of other amino acids, such as aspartate, that coincidentally increase ATP and possibly NADPH production (20). These metabolites are known to regulate mitochondrial function. Change in ATP/ADP ratio has been proposed to be the major determinant of the mecha-

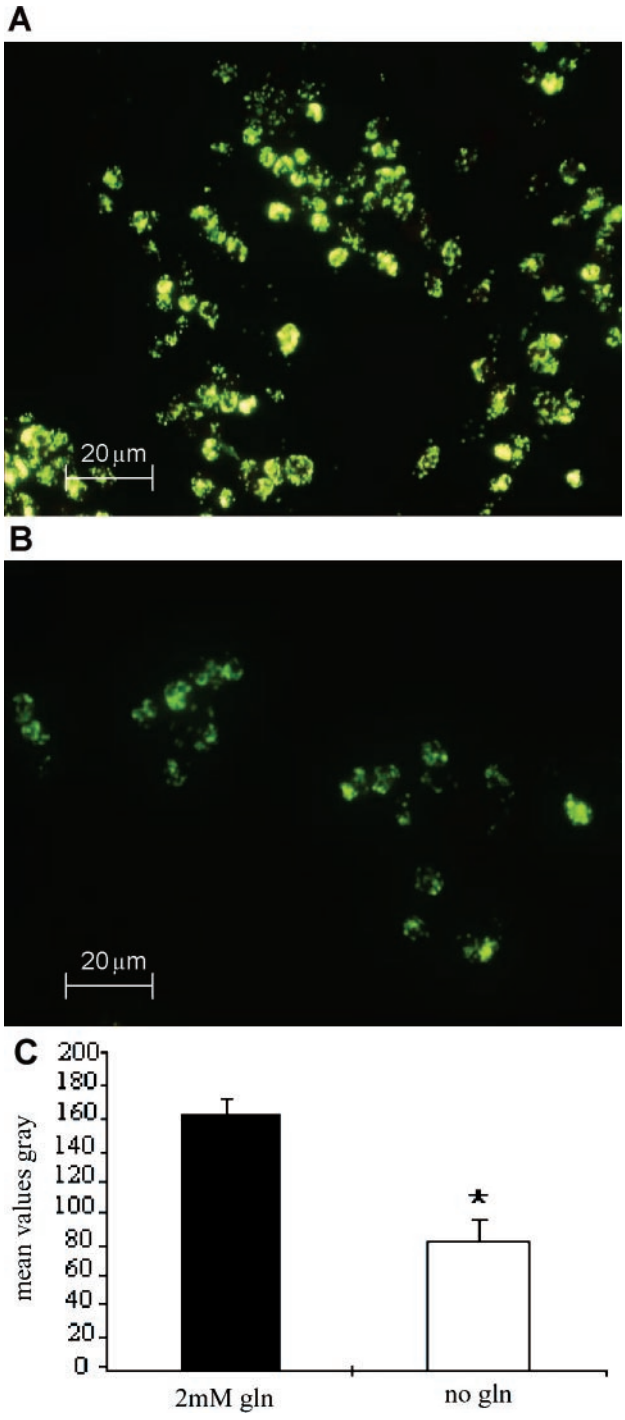


Fig. 6. Effect of glutamine (2 mM) on neutrophil phagocytosis analyzed by fluorescence microscopy using FITC-labeled *Escherichia coli*. Neutrophils were cultured for 6 h in the presence (*A*) or absence (*B*) of 2 mM glutamine. *C*: mean gray values ± SD of relative fluorescence intensity from the corresponding images of *A* and *B*.

nism of cell death. A moderate decrease of ATP level leads to apoptosis, whereas a marked decrease of this nucleotide causes necrosis (11, 15).

In certain pathological conditions such as sepsis, in which extracellular glutamine concentration is decreased (9, 14), the neutrophil may be subjected to accelerated rates of apoptosis, resulting in increased bacterial proliferation at sites of infection and, thus, enhanced levels of complement activation and subsequent tissue damage. The role of glutamine in enhancing and controlling inflammatory processes, including neutrophil activity, is thus critical to host defense (7).

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