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Phagocytosis of Nonapoptotic Cells Dying by Caspase-Independent Mechanisms[1]

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Caspase activation, exposure of phosphatidylserine (PS) on the outer surface of the plasma membrane, and rapid phagocytic removal of dying cells are key features of apoptosis. Nonapoptotic/necrotic modes of death occur independent of caspase activation, but the role of phagocytosis is largely unknown. To address this issue, we studied phagocytosis by human monocyte-derived macrophages (HMDM) and rat microglial cells. Target cells (Jurkat) were stimulated by several different methods that all caused caspase-independent death. First, we induced necrosis by combining toxins with ATP-depleting agents. Under these conditions, neither PS was exposed nor were such cells phagocytosed before their death. However, once the plasma membrane integrity was lost, the dead cells were rapidly and efficientl engulfed by HMDM. Next, we triggered Jurkat cell death with staurosporine in the presence of the pan-caspase inhibitor zVAD-fmk. Under these conditions, death occurred by delayed necrosis and without exposure of PS. Nevertheless, such lethally challenged cells were phagocytosed before the loss of membrane integrity. Finally, we triggered Ca^{2+} influ in Jurkat cells with an ionophore, or in neurons by glutamate receptor stimulation, respectively. In both models, PS was exposed on the cell surface. Ca^{2+}-stressed cells were phagocytosed starting at 30 min after stimulation. Protein kinase C inhibitors prevented Ca^{2+}-mediated PS exposure and phagocytosis. Essentially, similar phagocytosis data were obtained for all models with HMDM and microglia. We conclude that also cells dying nonapoptotically and independent of caspase activation may be recognized and removed before, or very quickly after, membrane lysis.

Apoptosis is an evolutionarily conserved process, essential in the shaping of organs during development and in the maintenance of tissue homeostasis in adult life (1). One of the most conspicuous features of apoptotic death is the efficien and swift removal of dying cells by professional macrophages or by neighboring cells (2–4). This process ensures the uptake of death-destined cells before their membrane lysis. The spillage of intracellular contents containing immunogenic Ags, proteases and other hydrolases, excitotoxins and substances disturbing cellular communication is thus prevented. In addition, phagocytosis of apoptotic cells appears to trigger signal pathways in macrophages fundamentally different from those elicited by uptake, e.g., of bacteria (5). Instead of a proinflammator state, the release of antiinflammator cytokines is triggered (6, 7). Taken together, these features are responsible for making apoptosis a silent type of death.

A variety of recognition molecules on the surface of apoptotic cells is important for the recognition and engulfment of the dying cells before membrane lysis. Parts of the genetic program for adhesion, uptake, and digestion by phagocytic cells seem to be genetically conserved from *Caenorhabditis elegans* to primates (8, 9). In the mammalian system, various recognition mechanisms have been described (3, 10, 11). It appears that a number of sequential processes similar to those of leukocyte rolling and sticking to endothelial cells or migration of cells (9, 12) are involved. Information on the specifi contribution of different mechanisms in various cell types and tissues is still sparse. Candidate macrophage receptors for apoptotic cells include CD36/vitronectin together with thrombospondin (3, 10, 13), lectins (14), pattern recognition receptors such as scavenger receptors (15, 16), and CD14 (5, 17, 18). Target cells may signal their death to phagocytes by exposure of certain carbohydrates (19) or of phosphatidylserine (PS)[3] (3) on the outer leafle of the plasma membrane (20). PS exposure on the surface of apoptotic cells has been closely associated with activation of caspases (21), although a specifi cleavage event has to date not been identified In fact, measurement of PS exposure has become one of the most widely used parameters to characterize apoptotic cell populations (22, 23). Both caspase activation (24) and PS exposure triggered by active caspases have been considered as indispensible hallmarks of apoptosis, and as prerequisites for phagocytosis (25, 26), although this position is still matter of ongoing debate (27, 28).

In particular in pathological settings, cell death frequently occurs independent of caspases and may often be nonapoptotic (29). Information on recognition and uptake of dying cells in such situations is sparse. Sometimes such cells may not be taken up by phagocytes as intact entities (30), and their remainders may only be removed after more excessive breakdown (31). However, it is unknown whether phagocytosis of necrotic cells is generally precluded. We addressed this issue by triggering three different types

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[3] Abbreviations used in this paper: PS, phosphatidylserine; ActD, actinomycin D; $[Ca^{2+}]_i$, intracellular calcium concentration; calcein-AM, calcein-acetoxymethyl ester; CGC, cerebellar granule cells; DEVD-afc, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin DiI, octadecyl (C_{18}) indocarbocyanine; EH-1, ethidium homodimer-1; GSNO, *S*-nitrosoglutathione; H-33342, Hoechst-33342; HMDM, human monocyte-derived macrophage; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclo-hepten-5,10-imine; PKC, protein kinase C; STS, staurosporine; zVAD-fmk, benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

of necrotic and caspase-independent cell death, and examined PS exposure and phagocytosis before and after membrane lysis. A special fluorescen assay to study phagocytosis of lysed cells was developed for this purpose.

Materials and Methods

Materials

Calcein-acetoxymethylester (calcein-AM), Hoechst-33342 (H-33342), octadecyl (C_{18}) indocarbocyanine (DiI), and ethidium homodimer-1 (EH-1) were obtained from Molecular Probes (Eugene, OR). FITC-conjugated annexin V was obtained from Boehringer Mannheim (Mannheim, Germany). (+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclo-hepten-5,10-imine (MK-801) came from RBI (Biotrend Chemikalien GmbH, Köln, Germany). *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumari (DEVD-afc) was from Biomol (Hamburg, Germany). Benzoyloxycarbonyl-Val-Ala-Asp-fluorometh ylketone (zVAD-fmk) was purchased from Bachem Biochemica GmbH (Heidelberg, Germany). Rottlerin and calphostin C were from Calbiochem (Bad Soden, Germany). All other reagents were from Sigma (Deisenhofen, Germany), Merck (Darmstadt, Germany), or Riedel-de Haen (Seelze, Germany).

Preparation of monocyte-derived macrophages

Human monocytes were isolated as described previously (32). In brief, 250 ml of peripheral venous blood was drawn from a single volunteer. Citrate (0.31% w/v) was used as an anticoagulant. The blood was diluted 1.6-fold with PBS (pH 7.4) before centrifugation at 220 × *g* at 20°C for 20 min. The cell pellet was layered on a Percoll gradient (ρ = 1.077 g/ml), and the interphase containing the PBMC was obtained following centrifugation at 800 × *g* for 10 min. Cells were washed twice in elutriation medium (PBS, 2% heat-inactivated human AB serum, 2 mM EDTA, 5 mM glucose (pH 7.4)) before countercurrent centrifugal elutriation of the cells using a J2-MC centrifuge equipped with a JE-6B rotor (Beckman, Fullerton, CA). The monocyte-containing fraction was obtained at a flo rate of 39 ml × min^{-1} and a rotor speed of 3000 rpm. Elutriated monocytes were plated at an initial density of 2 × 10^5 cells/well on 48-well culture dishes. After 60 min at 37°C, nonadherent cells were removed and the medium was exchanged for fresh RPMI 1640 containing 10% heat-inactivated human AB serum (Sigma) and antibiotics (penicillin, 5000 IU/ml; streptomycin, 5 mg/ml). Monocytes were differentiated to macrophages for 8–12 days in a volume of 1 ml/well on 48-well culture dishes (32). Medium was exchanged 5 days after plating and before experiments. Human monocyte-derived macrophages (HMDM) were used for experiments between day 7 and 12 after plating.

Preparation of microglial cells

Wistar rat pups (1–2 days postpartum) were decapitated and the cerebrum was transferred to a buffer containing NaCl (137 mM), KCl (5.4 mM), Na_2HPO_4 (250 μM), KH_2PO_4 (235 μM), glucose (5 mM), and saccharose (58 mM) (pH 6.5) on ice. Following removal of meninges, brains were cut three times in different directions using a tissue chopper (Bachofer, Reutlingen, Germany) set to cut 400-μm thick pieces. The chopped tissue was digested in the presence of trypsin (0.5%), DNase I (0.05%), and $MgSO_4$ (6 mM) for 5 min with subsequent trituration through a pipette tip to obtain a cell suspension. The mixed cell suspension was then maintained in Eagle's basal medium supplemented with 10% FCS and antibiotics in 75-cm^2 flask (Primaria 3072; Becton Dickinson, Heidelberg, Germany) at a density of three brains/flas with medium change every 3–4 days. After 10–14 days in culture, microglial cells were selectively detached by shaking at 150 rpm for 6 h. The supernatant was then transferred into FCS-coated 75-cm^2 flasks and microglial cells were allowed to adhere for 1 h. The supernatant was aspirated, adherent cells were removed by trypsinization, and resulting microglia were plated on 48-well dishes at a density of 2 × 10^5/well and used for experiments the following day. Purity was always >90%, as determined by routine staining with FITC-labeled lectin from *Bandeiraea simplicifolia* BS-I (33, 34).

Preparation and analysis of neuronal cultures

Murine cerebellar granule cells (CGC) were isolated as described (35, 36). Neurons were plated at a density of 0.25 × 10^6 cells/cm^2 and cultured in Eagle's basal medium (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FCS, KCl (20 mM), L-glutamine (2 mM), penicillin-streptomycin, and cytosine arabinoside (10 μM, added 48 h after plating). Neurons were used for experiments after 8 days in vitro without further medium change. PS staining of CGCs was performed as described (37, 38). In brief, cells grown on glass-bottom culture dishes were incubated with glutamate and inhibitors. After incubations, a mix of H-33342 (0.5 μg/ml) and EH-1 (0.3 μM) was added to the culture for 10 min, followed by washing of CGC and subsequent incubation with annexin V solution (1% in annexin V-binding buffer containing 100 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$) in the dark for 2 min. Stained cultures were washed in binding buffer, and EH-1, H-33342, and fluorescei fluorescence were visualized simultaneously by confocal microscopy using a Leica DM-IRB microscope connected to a TCS-4D UV/VIS confocal scanning system (Leica AG, Benzheim, Germany). Ca^{2+} measurements with the indicator fura-2, and internal calibration were performed exactly as described (37, 38) using video imaging on an MCID system from Imaging Reasearch (St. Catherine's, Ontario, Canada) equipped with a dage-72 (Dage-MTI, Michigan City, IN) camera and a computer-controlled filte wheel (Sutter, Novato, CA).

Jurkat cell culture and ATP levels

Jurkat cells (human T cell lymphoma, clone E6, ATCC No. TIB-152) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, antibiotics (penicillin, 10,000 U/ml; streptomycin, 10 mg/ml), and glutamine (2 mM). All experiments were performed in RPMI 1640 without serum. ATP depletion was achieved by incubating Jurkat cells for 45 min in serum- and glucose-free medium in the presence of 2.5 μM oligomycin (39, 40). ATP was measured luminometrically exactly as described (39, 41).

PS exposure

Jurkat cells were seeded in 96-well plates at a density of 50,000 cells/well. After challenge, medium was replaced by annexin V-binding buffer containing fluorescein-conjugate annexin V (1.3% v/v), H-33342 (1 μg/ml), and EH-1 (1 μM). After one wash, three microscopic field containing 100–200 cells each were counted, excluding necrotic cells (EH-1 positive) from scoring.

Flow-cytometric analysis

In some experiments, cells were resuspended in 25 μl annexin buffer containing FITC-conjugated annexin V (1.3% v/v). After 5 min, 175 μl annexin buffer containing propidium iodide (10 μg/ml) was added. The cell suspension was analyzed by flo cytometry using a FACScalibur, as described (Becton Dickinson) (42).

Necrotic and apoptotic triggers

For all experiments, Jurkat cells were incubated in RPMI 1640 medium without serum. Apoptosis was triggered by addition of staurosporine (STS, 1 μM, 2 h), actinomycin D (ActD; 2 μg/ml, 10 h), camptothecin (10 μM, 10 h), or anti-CD95 Ab (CH-11, 100 ng/ml, 3 h). Treatment with ionomycin was performed by incubating Jurkat cells in annexin V-binding buffer containing ionomycin (2 μM) to achieve high $[Ca^{2+}]_i$ conditions. Two types of delayed STS-triggered necrosis were induced. First, Jurkat cells were treated with STS in the presence of zVAD-fmk, as reported recently (43). Second, Jurkat cells were treated with STS under conditions of ATP depletion, as described previously (39, 44). NO-induced necrosis was achieved by incubating the cells in serum- and glucose-free medium containing pyruvate (2 mM) plus *S*-nitrosoglutathione (GSNO, 0.4 mM), as described (44), in the presence or absence of ActD (2 μg/ml) or camptothecine (10 μM). Quantificatio of apoptosis and necrosis was routinely performed by staining with a mixture of the cell-permeant chromatin dye H-33342 (blue, 0.5 μg/ml) and the membrane-impermeant dye SYTOX (green, 0.5 μM) (39). The percentage of necrotic cells (SYTOX-positive, noncondensed nuclei), early apoptotic (intact plasma membrane, condensed chromatin), and late apoptotic (SYTOX-positive, condensed, or fragmented nuclei) was determined by scoring 300–500 cells in three to six different microscopic field using a Leica microscope and lenses providing ×400 fina magnification In some experiments, the membrane-impermeant dye EH-1 (red) was used instead of SYTOX (39).

Caspase activity

DEVD-afc cleavage activity was analyzed as described (45, 46) by lysing 2.5 × 10^5 Jurkat cells in a buffer containing HEPES (25 mM, pH 7.5), $MgCl_2$ (5 mM), EGTA (1 mM), Triton X-100 (0.5%), leupeptin (1 μg/ml), pepstatin (1 μg/ml), aprotinin (1 μg/ml), and PEFA-block (1 mM). The lysates were transferred to a microtiter plate, and the fluorometri assay was performed with a substrate (DEVD-afc) concentration of 40 μM. DEVD-afc cleavage was monitored with an excitation wavelength of 390 nm and emission wavelength of 505 nm. The activity was calculated using calibration curves generated with free afc. One unit was define as formation of 1 pmol afc.

Labeling of target cells for phagocytosis assays

Cells were stained with Fast Blue (1 μg/ml; Sigma) 1 day before treatment for at least 6 h. The cells were washed, and kept in fresh medium overnight. For some experiments, Jurkat cells were stained at the end of the toxic stimulation with 2 μM calcein-AM for 20 min. The dye was then removed by washing in medium before cells were added to macrophages.

Phagocytosis assay

Phagocytic cells (HMDM or microglia) were stained for 20 min with DiI (2.5 μg/ml) to visualize cell bodies. Jurkat cells (prelabeled with Fast Blue) were added to macrophages at a ratio of 10 (target):1, and phagocytosis was allowed to proceed for 1 h at 37°C. Nonphagocytosed target cells were then removed by fiv extensive washing steps in PBS or by exposure to trypsin (0.005% w/v) for 3 min at 37°C, and subsequent washing. Ingested cells were counted by their blue (Fast Blue) fluorescenc in three to four microscopic field containing 40–80 phagocytes each. Phagocytic index was calculated by multiplying the percentage of phagocytosing cells with the average of ingested cells per phagocyte. All experiments with nonlysed target cells were performed in parallel with a different labeling strategy yielding essentially similar results: Jurkat cells were labeled with calcein-AM, as described, and used for the phagocytosis assay. This method allowed to confir the maintenance of membrane integrity during the phagocytosis assay (necrotic cells lose calcein). Moreover, phagocytosed cells frequently seemed to be broken up in macrophages when left for more than 1 h. Then calcein spread throughout the macrophage, indicating that the target cell had really been ingested and not just adhered to the phagocyte.

Western blot analysis

The release of cytochrome *c* from Jurkat cell mitochondria was analyzed as described before (44, 47). At the indicated time points, Jurkat cells (1×10^6) were harvested and fractionated in cytosol and remaining organelles. Protein from the supernatants was separated on 15% polyacrylamide gels. Cytochrome *c* was detected by the enhanced chemoluminescence (ECL) reaction after blotting on nitrocellulose membranes with a mAb raised against pigeon cytochrome *c* (clone 7H8.2C12; PharMingen, San Diego, CA).

Statistical analysis

Phagocytosis experiments were run in quadruplicates and repeated in at least three cell preparations. Scoring was performed by two different observers blinded to the experimental conditions. Statistical significanc was evaluated from the original data using Student's *t* test. The Welch test was applied when variances were not homogenous within the compared groups. A *p* value of less than 0.05 was considered to be significant

Results

Monitoring of phagocytosis and the mode of death

To analyze phagocytosis of cells dying by different modes, we designed a multiparameter fluorescen phagocytosis assay. Macrophages were labeled with the lipophilic tracer DiI, which was bright, well retained, and did not disturb phagocytosis function. Target cells were labeled with Fast Blue, a dye of very low toxicity that has previously been used as a neuronal tracer in vivo (48, 49). Fast Blue-labeled cells retained the tracer dye even when the plasma membrane was lysed (Fig. 1*A*). Chromatin condensation was quantitated in parallel cultures by staining with H-33342 (39). The data were confirme by Fast Blue staining, which proved to be a good qualitative indicator of the chromatin state of target cells in the phagocytosis assay (Fig. 1*A*). In some experiments, loss of membrane integrity of individual cells was additionally monitored by using either the red fluorescen dye EH-1 or the green fluores cent dye SYTOX, which selectively stained nuclei of cells with a lysed plasma membrane (Fig. 1*A*). To evaluate the phagocytosis assay, we compared uptake of control Jurkat cells and STS-challenged apoptotic cells. Only the apoptotic cells were taken up and were clearly visible as blue spots within phagocytic vacuoles of red macrophages (Fig. 1*B*).

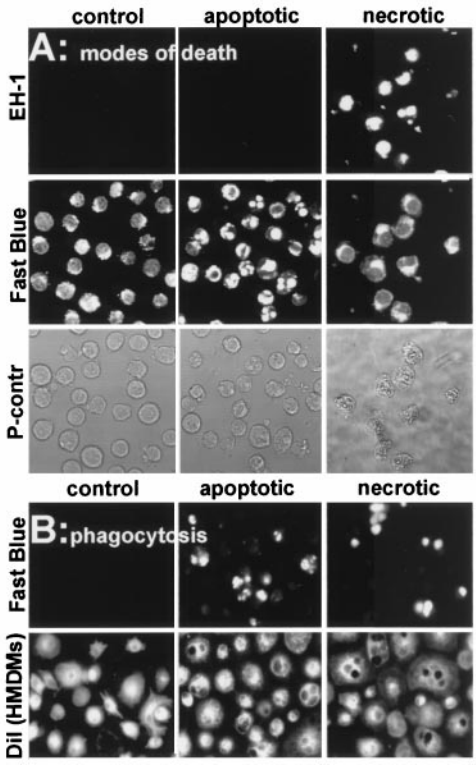

FIGURE 1. Fluorescent phagocytosis assay for necrotic cells. *A*, Jurkat cells were stained with Fast Blue and then separated into three pools. Cells were either left untreated (control) driven into apoptosis with STS (1 μM) or into necrosis under ATP-depleted conditions with oligomycin (2.5 μM) plus STS. Cell membrane integrity was probed by staining with EH-1 shortly before simultaneous confocal imaging of the cells in two fluores cent channels (Fast Blue, EH-1) and the polarization contrast mode (P-contrast). The width of one image panel corresponds to 75 μm. *B*, HMDM were prestained with DiI. DiI labeled all intracellular membranes and the plasma membrane and emitted red fluorescen light (λ_{em} = 586 nm). Jurkat cells were prestained with Fast Blue, triggered to undergo apoptosis or necrosis as in *A*, and then added as target cells to the HMDM. After 30 min, nonphagocytosed Jurkat cells were removed by washing and mild trypsinization. Two digital images of a given microscopic fiel were obtained with different filte settings (DiI and Fast Blue). Localization of engulfed target cells within phagocytic vacuoles is evident. The width of one image panel corresponds to 150 μm.

Deficien uptake of prenecrotic cells during STS-induced death in ATP-depleted cells

STS-triggered apoptosis of Jurkat cells can be diverted to necrosis, when experiments are performed under conditions of ATP depletion (39, 40, 50). To start our examinations, we used this well-characterized model system to produce different modes of cell death. Jurkat cells challenged with STS under normal metabolic conditions (ATP high) activated caspases (DEVD-afc cleavage), condensed their chromatin (>85% of the cells), and exposed PS (annexin V positive) within 1–2 h. Plasma membrane integrity was retained for 4–5 h. When such cells were coincubated with HMDM after 2 h of STS exposure, they were efficientl phagocytosed (Fig. 1). Similar correlations of apoptotic changes, PS exposure, and efficien phagocytosis of nonlysed cells were obtained when cells were challenged with ActD, camptothecin, or agonistic Abs against CD95 (not shown).

When cells were challenged with STS in the absence of glucose (low ATP) and in the presence of oligomycin, an inhibitor of the mitochondrial ATP synthase, they died necrotically (membrane

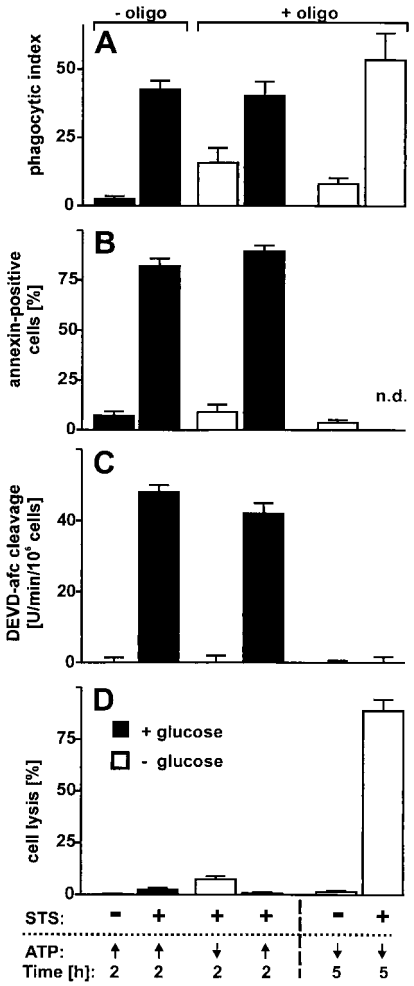

FIGURE 2. Phagocytosis of lysed necrotic cells. Jurkat cells were grown in the absence or presence of glucose (■, 10 mM) or absence or presence of oligomycin (oligo, 2.5 μM). For clarity reasons, the corresponding cellular ATP content is indicated by upward arrows (ATP > 90% of control cells in glucose-containing medium) and downward arrows (ATP < 15% of control). Cells were treated with STS (1 μM) for various periods of time. Then *A*, phagocytic uptake; *B*, the number of PS-exposing cells; *C*, caspase activity (DEVD-afc cleavage); and *D*, the percentage of cells with lysed plasma membrane (staining with vital dye SYTOX) were quantitated. All data are means ± SD of three experiments. N.d.: not determined. Reduction of phagocytosis of STS (2-h)-treated cells by oligomycin (third bar) was statistically significan ($p < 0.05$) when compared with cells treated with STS alone or STS + oligo + glucose. Phagocytosis was not significantl ($p > 0.05$) reduced after 5 h of treatment (6. Bar).

lysis) after 4–5 h. Compared with ATP-adequate cells, caspase activity, chromatin condensation, and PS exposure were completely blocked in ATP-depleted, prenecrotic cells 2 h after STS challenge (Fig. 2) and did not increase until the cells lysed. Such prenecrotic cells were not significantl phagocytosed (Fig. 2*A*). Notably, in our experimental system, oligomycin did not directly inhibit PS exposure or phagocytosis, as shown in other systems (28, 51): cells treated with STS plus oligomycin in the presence of glucose (high ATP) behaved exactly like cells treated with STS alone (Fig. 2). Those experiments corroborated the hypothesis that apoptotic cells are taken up by macrophages, while prenecrotic ATP-depleted ones are not.

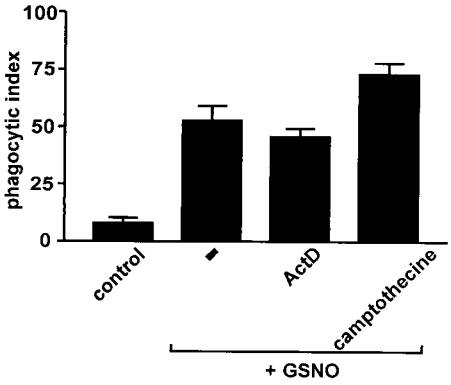

FIGURE 3. Phagocytosis of Jurkat cells after induction of NO-tiggered necrosis. Jurkat cells were treated in glucose-free medium with GSNO (0.4 mM) in the presence or absence of ActD (2 μg/ml) or camptothecine (10 μM). After 24 h, >95% of the cells under all conditions were necrotic (noncondensed chromatin, SYTOX positive). Those cell populations were used in a phagocytosis assay with HMDM, and were compared with untreated control cells. Data are means ± SEM of triplicate determinations.

Phagocytic uptake of ATP-depleted necrotic cells

When following the fate of the target cells throughout the phagocytosis assay, we occasionally observed that some of the cells losing their membrane integrity prematurely during the assay time were engulfed by macrophages. Therefore, we tested what would happen, when we used cells in the phagocytosis assay, that all had broken plasma membranes. A population of pure necrotic cells was obtained by exposure to STS under ATP-depleting conditions for 5 h (Fig. 2*D*). Such late necrotic cells were efficientl taken up by macrophages (Fig. 1*B*). Most of the necrotic cells appeared to be engulfed as single entities, similarly to the early apoptotic cells (Fig. 1*B*). Phagocytosis of necrotic Jurkat cells killed by STS was as efficien as the one of early apoptotic cells (Fig. 2*A*). This implies that in certain types of necrosis, cells do not necessarily have to disintegrate before their remnants are removed. Rather, phagocytosis seems to be possible very quickly after the loss of plasma membrane integrity.

We tested whether this phenomenon was also observed when cells were killed independently of STS by the physiological NO donor GSNO. In glucose-free medium, this substance triggered >85% necrosis within 24 h (41), independent of whether it was used alone or in combination with the chemotherapeutics ActD and camptothecine. Cells treated in the described manner were offered to HMDM for phagocytosis (Fig. 3). Also in this model, necrotic Jurkat cells were efficientl phagocytosed once they had lost plasma membrane integrity. These results suggest that phagocytic uptake of necrotic cells may occur under different pathological settings.

Phagocytosis of PS-negative prenecrotic cells after long-term exposure to STS

Inhibition of caspases has previously been shown to switch death in lymphoid cells from apoptosis to necrosis (52, 53). In Jurkat cells, STS-triggered apoptosis was switched to delayed necrosis in the presence of the pan-caspase inhibitor zVAD-fmk (43). We used this model to further study phagocytosis in caspase-independent, nonapoptotic cell death. After 24-h stimulation, such cells were efficientl phagocytosed (Fig. 4*A*), although they did not expose PS (Fig. 4*B*). At that stage, cells had no condensed chromatin, no significan caspase activity, adequate ATP levels (>70% of

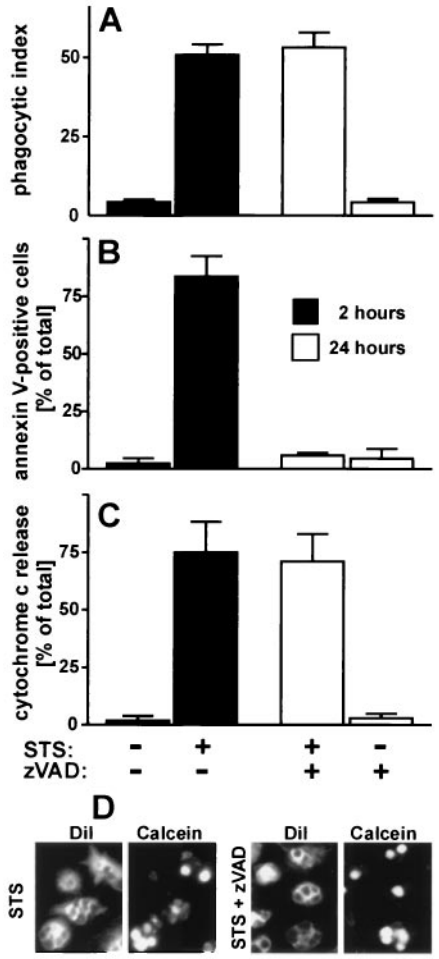

FIGURE 4. Phagocytosis of PS-negative nonapoptotic cells. Jurkat cells were incubated either with STS (1 μM, 2 h) as apoptotic control or with STS plus zVAD-fmk (50 μM, 24 h), and then added to phagocytes. Under all conditions, less than 10% of the cells had lost membrane integrity (EH-1 stain). *A*, After 1 h of interaction with HMDM, phagocytic index was scored microscopically. *B*, Jurkat cells were labeled with fluorescein-con jugated annexin V, and PS-exposing cells were scored by microscopy. Data in *A* and *B* are means ± SEM of three independent experiments. *C*, Cytochrome *c* release was analyzed by immunoblotting cytosolic fractions of Jurkat cells. Two blots were analyzed by densitometry, and mean values and range are shown. For quantitation, the amount releasable by 0.5% Triton X-100 was set to 100%. *D*, Jurkat cells were incubated with STS or zVAD plus STS as in *A* and used as target cells in the HMDM phagocytosis assay. Jurkat cells were labeled with calcein and HMDM with DiI. Fluorescence from each microscopic fiel was recorded with two selective filte settings. Colocalization of Jurkat cells with phagocytic vacuoles is evident.

control), and an apparently intact plasma membrane, although cytochrome *c* had been released from mitochondria to the cytosol (Fig. 4*C*). The caspase inhibitor in the culture supernatant was still functionally active (54; data not shown).

When cells were incubated for 48 h in total with STS plus zVAD, large-scale (>90%) necrosis was evident. Therefore, we took care to examine whether plasma membranes may have broken prematurely during our phagocytosis assay (after 24 h), transforming the prenecrotic cells to necrotic cells. For this purpose, we modifie the phagocytosis assay, by prelabeling the target cells with calcein-AM. This dye is only accumulated in cells with intact plasma membrane, and is lost rapidly upon lysis. Use of this assay yielded similar results as counting the uptake of Fast Blue-labeled cells, i.e., the target cells retained calcein throughout the phagocytosis assay and distinct calcein-containing target cells were detected within macrophages (Fig. 4*D*). These data suggest that some types of nonapoptotic cells may be engulfed by phagocytes before membrane lysis. The recognition mechanism appears to be independent of PS exposure.

Exposure of the phagocytosis marker PS in Ca^{2+}-triggered cell death

PS exposure has been generally considered to be an event controlled by caspase activation and specifi for apoptotic death (21, 55, 56). However, apart from caspases, ionophore-mediated increases of $[Ca^{2+}]_i$ have been shown to constitute an independent signal to trigger directly PS exposure and phagocytosis signaling (25). We hypothesized that PS exposure should occur before membrane lysis when necrotic/nonapoptotic demise was triggered via disturbance of the Ca^{2+} homeostasis. To test the hypothesis, we challenged Jurkat cells with the Ca^{2+} ionophore ionomycin. Indeed, cells became annexin V positive within 15–30 min (Fig. 5*A*), and microscopic analysis was corroborated by flow-cytometri analysis (Fig. 5*B*). When PS was exposed, the chromatin retained normal decondensed structure (Fig. 5*C*), and after 2–3 h, cells lysed without evident apoptotic morphological changes or DNA fragmentation (not shown) or any caspase activation (Fig. 5*D*). The caspase independence of ionomycin-triggered PS exposure was also tested in a further experimental system. Cells were treated in glucose-free medium with STS and oligomycin for 2 h. Under such conditions, caspase activation is entirely prevented (see Fig. 2*c*). Nevertheless, treatment of such cells with ionomycin triggered PS exposure (70–80% of the cells after 30 min).

Neither high concentrations of the caspase inhibitor zVAD-fmk (up to 100 μM), nor treatment with oligomycin inhibited ionomycin-triggered PS exposure. However, PS exposure due to ionomycin was completely blocked by the protein kinase C (PKC) inhibitors rottlerin and calphostin C. This suggests that distinct signaling pathways different from caspase activation contribute to PS exposure in Ca^{2+} ionophore-triggered necrosis. Thus, there seem to be mechanisms allowing PS exposure also in caspase-independent modes of death.

Similar events were observed in CGCs treated with lethal concentrations (100 μM) of the excitotoxic neurotransmitter glutamate. Such excitotoxic death has some features of apoptosis and some of necrosis (57, 58). At 30–60 min after triggering of Ca^{2+} influ via the glutamate-controlled *N*-methyl-D-aspartate receptor, most of the cells (>90%) had translocated PS. The Ca^{2+} concentrations remained high, nuclei condensed within 60–90 min (Fig. 6), and plasma membrane integrity was retained for 5–7 h. Cell death, chromatin condensation, and PS exposure occurred independent of the activation of any known caspase, since they were not affected by up to 100 μM zVAD-fmk (Fig. 6). All toxic events triggered by glutamate, including Ca^{2+} influx were prevented by preincubation with the noncompetitive *N*-methyl-D-aspartate receptor antagonist MK-801. Taken together, these data suggest that increased $[Ca^{2+}]_i$ may trigger PS exposure in cells dying by apoptosis or necrosis without necessitating caspase activation.

Phagocytosis of PS-positive nonapoptotic cells

PS exposure may either be sufficien alone for phagocytic uptake of cells (26, 59) or be an indicator of other cell surface changes important for recognition by phagocytes. Both possibilities imply that ionomycin-challenged, annexin V-positve cells may be recognized and engulfed by macrophages before lysis of their plasma membrane, although they were nonapoptotic. We tested this hypothesis by offering ionomycin-treated Jurkat cells to HMDM for ingestion. A significan uptake occurred compared with nontreated

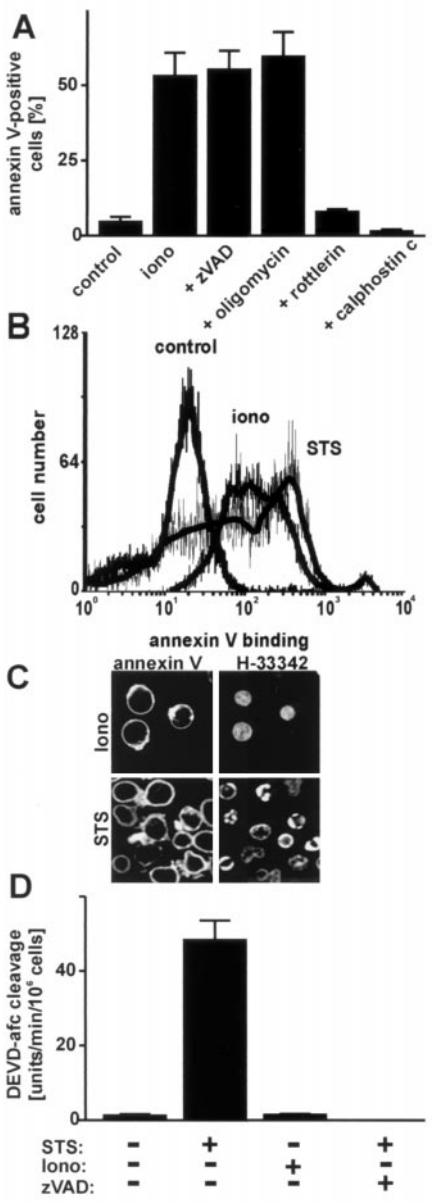

FIGURE 5. Caspase-independent exposure of PS in lymphoid cells. *A*, Jurkat cells were treated with 2 μM ionomycin (iono) in the presence or absence of zVAD-fmk (zVAD, 50 μM), calphostin C (5 μM), rottlerin (5 μM), oligomycin (2.5 μM), or solvent alone. Inhibitors were added 30 min before the stimulus, and after 30 min of incubation with iono, the number of annexin V-positive cells was determined. Data are means ± SEM of three independent experiments. *B*, Jurkat cells, as treated in *A* with or without ionomycin, were analyzed by flo cytometry. For comparison, cells treated for 90 min with STS were used. Propidium iodide-positive cells were gated out, and representative distributions of annexin V-fluorescei fluorescenc are shown. *C*, Jurkat cells were treated with ionomycin (2 μM, 30 min) or STS (1 μM, 2 h), and stained with H-33342, EH-1, and annexin V before they were imaged by confocal microscopy. All cells were EH-1 negative (not shown). *D,* Caspase activity (DEVD-afc cleavage) was determined 30 or 90 min after stimulation (similar results). As positive and negative controls, cells were treated with STS (1 μM, 2 h) or with STS plus zVAD-fmk (50 μM, 24 h). Values represent means ± SEM of triplicate experiments.

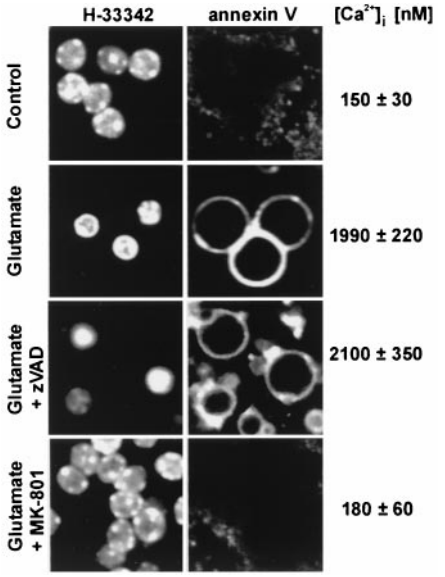

FIGURE 6. PS exposure in caspase-independent excitotoxic neuronal death. CGC were stimulated with glutamate (100 μM) in the presence of zVAD-fmk (zVAD, 100 μM), MK-801 (2 μM), or solvent alone. After 90 min, cells were stained with H-33342 (chromatin structure), EH-1 (not shown, ≥95% of cells negative), and FITC-labeled annexin V. Confocal microscope images of the fluorescen labels are shown. In parallel cultures, $[Ca^{2+}]_i$ was measured by video microscopy ratio imaging of fura-2. Data are means ± SD from 20 individual cells in three different experiments.

control cells (Fig. 7*A*). Notably, the targets were engulfed with still intact plasma membrane, as they retained calcein during the phagocytosis procedure.

Compared with STS-treated apoptotic cells, ionomycin-challenged Jurkat cells became about 50% less strongly annexin V positive (Fig. 5*B*). In parallel, the phagocytic index for the ionomycin cells was only half as high. A correlation of PS exposure with the phagocytic index was also observed, when target cells were pretreated with calphostin C or zVAD: the caspase inhibitor had no significan effect on phagocytosis/PS exposure, while calphostin C pretreatment was significantl inhibiting. In cells in which STS-dependent caspase activation was inhibited by ATP depletion with oligomycin, exposure to ionomycin was still able to trigger phagocytosis (phagocytic index = 35 ± 5% with ionomycin; 15 ± 3% without ionomycin). All of these experiments indicate that PS exposure correlates well with phagocytosis of ionophore-treated cells.

To distinguish between adhesion and ingestion of nonapoptotic cells by macrophages, we used two methods. First, only adherent cells were detached by trypsinization, and the large number of remaining target cells indicated real engulfment. Second, we labeled them with calcein and followed the fate of the dye. After prolonged (90-min) exposure of calcein-labeled Jurkat cells to HMDM, macrophages were observed that stained all green (Fig. 7*A*), instead of just containing distinct green target cells (see Fig. 4*D*). After 3 h, 50% of the macrophages were green of calcein in the cytoplasm. This can best be explained by release of the fluo rescent label from the target cell into the macrophage after complete phagocytosis and initiation of digestion.

Phagocytosis by microglial cells

In a fina set of experiments, we investigated whether genuine tissue macrophages were also able to ingest nonapoptotic cells, and we chose microglial cells, the professional neural phagocytes. Jurkat cells were treated as described above either with ionomycin to induce caspase-independent PS exposure, or with STS in the presence of zVAD-fmk, or with STS under conditions of low cellular ATP levels, to induce a prenecrotic state. Apoptotic control cells (STS exposure) were substantially ingested by microglia

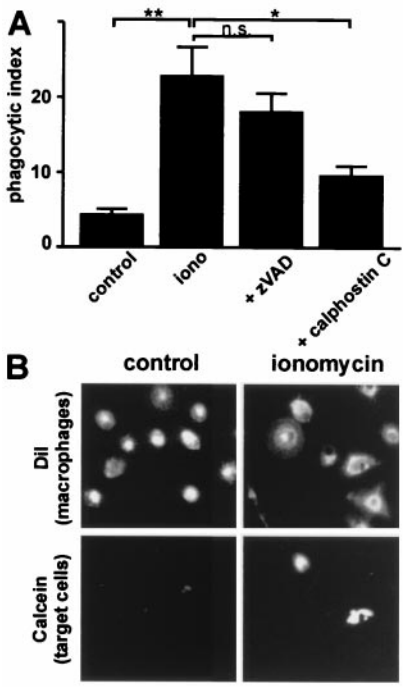

FIGURE 7. Phagocytosis of PS-positive nonapoptotic cells. Jurkat cells were stimulated with ionomycin (2 μM) in the presence of zVAD-fmk (zVAD, 50 μM), calphostin C (5 μM), or solvent alone. Then, Jurkat target cells were added to HMDM for phagocytosis assays. *A*, Phagocytic index was evaluated by scoring of microscopic images of DiI-labeled macrophages and Fast Blue-labeled target cells. Values are means ± SEM of three experiments. *, $p < 0.05$; **, $p < 0.01$; n.s., not significan ($p > 0.05$). *B*, Jurkat target cells were labeled with calcein instead of Fast Blue. Both fluorescence were recorded from HMDM cultures exposed to untreated target cells (control) or cells treated with ionomycin (2 μM). Some macrophages are shown to be labeled by calcein after the phagocytosis assay, due to the uptake of calcein-labeled target cells.

(Fig. 8). Ionomycin-treated cells were phagocytosed to a lesser extent, whereas ATP-depleted, prenecrotic cells failed to be taken up. Treatment of Jurkat cells with zVAD-fmk and STS facilitated a significan uptake by microglial cells, but to a lesser extent than

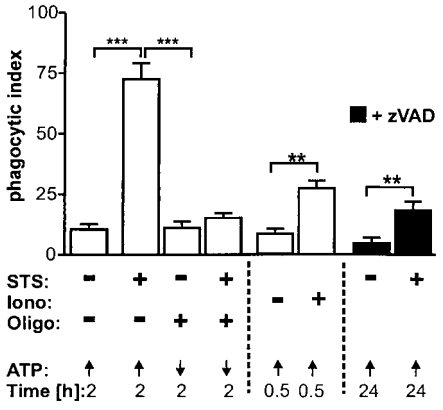

FIGURE 8. Phagocytosis by microglia. Jurkat cells were kept at conditions of high (arrows up) or low (arrows down) ATP content, as described in Fig. 2. They were treated either with ionomycin (2 μM) or STS in the presence or absence of zVAD (zVAD-fmk, 50 μM) for the times indicated. Then they were offered to rat microglial cells for phagocytosis. Phagocytic index was evaluated by microscopy. Values are means ± SEM of three to four experiments.

observed with HMDM. These experiments essentially confirme that there may be multiple situations and pathways for recognition and uptake of cells that do not die by classical apoptosis and fail to activate caspases.

Discussion

A number of elegant studies have shown that cells dying by apoptosis are specificall recognized and taken up by macrophages before their plasma membrane lyses (3, 7, 10, 60). Similar information is lacking on nonapoptotic modes of cell death. We provided in this study initial evidence that necrotic cells can be effi ciently recognized by macrophages, and be taken up before or shortly after lysis of their plasma membrane. To associate the mode of cell death with subsequent phagocytosis, we applied a combination of different fluorescen stains to characterize the type of demise in individual cells. For the classification we kept to the following well-characterized (29, 39, 40, 47, 61–63) and pragmatic approach: Necrosis was define by plasma membrane permeability to the fluorescen chromatin dyes EH-1 or SYTOX in cells with noncondensed chromatin. Early apoptotic cells were define by their typically condensed chromatin, and the capacity to exclude EH-1 or SYTOX. Late apoptotic cells had condensed chromatin and broken plasma membranes, and were not used in this study. In addition, we introduced in this study the term prenecrotic to indicate cells that had been exposed to a necrotic stimulus, but still had an intact plasma membrane and normal chromatin.

In addition to the characterization of cell death, we further utilized a combination of dyes to label macrophages and target cells in a fluorescen phagocytosis assay. The main technical problems with many candidate dyes were the diffusion from one cell type to the other, and the potential loss of the label after membrane lysis. With respect to these problems, Fast Blue proved to be an optimal solution to label target cells. An additional advantage of the stain for this study was that it indicated changes of the chromatin structure. Although it was distributed throughout the cell in untreated Jurkats, it was very strongly accumulated in apoptotically condensed chromatin lumps, and allowed identificatio of the mode of death of cells directly used in the phagocytosis assay. Moreover, the study of different stages and modes of cell death was further facilitated by the addition of calcein-AM or of SYTOX. This allowed the identificatio of membrane intactness of individual cells during the phagocytosis experiment.

A notorious problem of all phagocytosis assays is the distinction of cells that adhere to macrophages from those that have been engulfed. Even though we used confocal microscopy, the question could not be solved unambiguously. We used two alternative approaches: firs we introduced a trypsinization step, to detach all target cells that only bound to macrophages, but had not been taken up. The macrophages themselves were extremely trypsinization resistant after 8–12 days of differentiation on plastic. The large number of target cells remaining after trypsinization indicated that real phagocytosis had occurred. Second, we followed the fate of cells labeled with calcein-AM. Calcein-AM is converted to the fluorescen and membrane-impermeant dye calcein within live cells. Such cells retained the dye initially after being taken up into macrophages. At later stages, macrophages were observed that stained with calcein all over, indicating that the dye had been released into the phagocyte after uptake of the target cell. Taken together, these data suggest that mechanisms exist that allow the recognition and phagocytosis of prenecrotic cells by professional macrophages.

Quantitative phagocytosis assays would have been hardly possible in this study without the availability of experimental models that yielded a population of cells undergoing a well-characterized necrotic death with predictable and relatively synchronous kinetics. Thus, we were able to compare effects on a single cell line in three different necrotic models and in apoptosis triggered by four stimuli. Interestingly, necrotic cells seem to generate phagocytosis recognition signals in diverse ways, and efficienc of phagocytic uptake seems to be largely different between the models we used: 1) The absence of any PS exposure or phagocytic uptake of prenecrotic cells in the ATP-depletion model corroborates the frequently expressed view on the absence of any recognition mechanism in necrotic cells (6, 11, 64). However, it is remarkable in this context, that cells were recognized and taken up by macrophages relatively soon after lysis of their membrane. This may not prevent spillage of some intracellular contents from such dying cells, but necrotic cells may be removed quickly from tissues under certain conditions to allow immediate reorganization. 2) In the caspase-inhibition model, prenecrotic cells were phagocytosed in the absence of PS exposure. This would imply that PS exposure is not an indispensable requirement for phagocytosis of prenecrotic cells to occur. Also, in apoptotic cells, a number of different recognition mechanisms have been identifie (3, 10), and it may depend on the cell types whether PS exposure is sufficient necessary, or irrelevant for the engulfment by phagocytic cells (17, 26, 59, 65–70). 3) The ionomycin model showed that PS exposure is not specifi for apoptotic cells. It has been demonstrated earlier that inihibition of the aminophospholipid translocase by thiol-reactive agents would trigger PS exposure, and that such a mechanism may explain PS exposure in necrotic cells (71). Moreover, calcium has been implicated as a signal for PS exposure in apoptotic death and possibly in physiological situations (25, 72, 73). In this study, we complement this information by showing that PS exposure can occur in calcium-triggered necrotic death, even in the presence of high concentrations of the pan-caspase inhibitor zVAD, and that such cells are recognized and taken up by macrophages before membrane lysis. This model may have a correlate in a number of pathological settings, in which calcium has been suggested to be a key mediator of necrotic death (74, 75).

Calcium has also been implicated in apoptotic death (29, 74). Therefore, it was important to consider the possibility that ionomycin-triggered PS was in fact an apoptotic response. The mode of death triggered by calcium seems to depend on the intensity of insult (61, 63, 76), and on costimulatory signals. For instance, the combination of phorbol ester and ionomycin triggers apoptosis in lymphoid cells (77, 78), while ionomycin alone rather seems to inhibit apoptosis elicited by other stimuli (78, 79). We found in this study that ionomycin triggered clear necrosis. Thus, this experimental system also showed that phagocytosis can occur in caspase-independent modes of cell death that occur without any morphological indication of apoptosis.

Uptake of necrotic cells did obviously not correlate with PS exposure in different experimental systems, although we found that it may occur in special cases (Ca^{2+} stress). Some mechanistic considerations appear to be interesting with respect to future studies: 1) Neither PS, nor other structures that would allow recognition by macrophages, seem to be exposed in the ATP-depletion models. Possibly energy-requiring steps are necessary, as for other apoptotic processes (63). However, necrotic cells were taken up after rupture of the plasma membrane. At present, we cannot exclude that this mechanism may involve recognition of PS by macrophages. Membrane asymmetry with respect to PS may be lost rapidly in necrotic cells, and the methods available to us did not allow us to characterize this process. 2) Earlier experiments in neurons (37, 54, 57) and lymphoid cells (21) suggested that PS exposure requires caspase activation. Although this holds true under many experimental conditions, evidence has also been provided that either cell type may become annexin V positive even when caspases are inhibited (25, 57). In this study, we provided additional evidence that there may be at least two different pathways of PS exposure, one inhibitable by zVAD, and one inhibitable by PKC inhibitors, but not by zVAD. The inhibitor pattern suggested some specificit for the δ isoform of PKC, and PKC δ has before been associated with various aspects of apoptosis (80–84). It may be interesting to follow the role of PKC in necrotic PS exposure, but more extensive studies are needed to really identify signal transduction pathways and relevant molecular switches. We focused in this study rather on the question on whether recognition by macrophages may also be regulated by different drugs after different stimuli. In fact, inhibition of ionomycin-triggered PS exposure by calphostin C, but not by zVAD, correlated with the effect of these different drugs on recognition and uptake of cells by macrophages.

A well-accepted dogma in cell death research has been the failure of phagocytes to remove necrotic cells. According to this scenario, lysing cells would constitute a danger for triggering inflam matory responses. A recent report showed that apoptosis was converted to necrosis in the interdigital space of mice either treated with caspase inhibitors or lacking *apaf-1*. Although phagocytosis of apoptotic cells could not occur in this study, no signs of inflam mation were detected and finger developed normally (30). This report suggests that there may be ways for a clean, silent removal of nonapoptotic cells. Our data expand this view by showing that there may be different ways and mechanisms for uptake of prenecrotic cells.

Apoptotic cells may under various circumstances also be ingested by neighboring cells instead of professional macrophages (3, 85, 86). From experiments in mice, it is known that in livers damaged by TNF, uptake of apoptotic hepatocytes is often a very conspicuous feature, while necrotic hepatocytes seem to remain untouched until their debris is taken up by invading leukocytes (31). This may constitute a major difference between apoptotic and necrotic death in certain tissues. In addition, the reaction and fate of phagocytes may be different after ingestion of apoptotic, necrotic, or microbial targets (5, 87, 88).

Different ways of recognition and uptake of dying cells may have a major implication on immunological parameters. It has been claimed for several years that presentation of Ags from apoptotic cells may represent a mechanism of initiation of autoimmune disease (89). It has also been suggested that different modes of cell death have an effect on systemic elimination of trypanosomes (90), on the maturation of dendritic cells (91), on Ag presentation by dendritic cells (91), and on macrophage antitumor activity (92). In most of those studies, the complexity of necrotic cell death has been avoided by the use of freeze-thawing as standard method to induce necrotic cells. Our study shows that the exact mode of necrotic death has an important impact on the recognition by phagocytes. This suggests that different forms of necrosis may alter major immunological reactions, such as autoimmunity, tumor regulation, and triggering of the T cell response.

During the last years, it has become evident that apoptosis and necrosis are not fundamentally different modes of cell death, but that in many instances, they may share initiation, signaling, and execution mechanisms (29, 58). Therefore, it has been difficul to fin unambiguous biochemical or functional markers for the mode of cell death. The most clearly distinguishing features of apoptosis were the activation of caspases, and the selective uptake by phagocytes. Our data now show that phagocytosis is principally possible

also in caspase-independent cell death. This may be important for many pathological situations, in which cell death frequently occurs by necrosis (29), and for chemotherapy, in which nonapoptotic death has been discussed to play an important role (93). Evidence for phagocytosis of necrotic cells supports the teleological view that mammalian organisms, in which cellular demise is less strictly dependent on caspases than in *C. elegans*, may have developed ways and means to efficientl remove apoptotic and necrotic cells, without major damage to the organism.

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