Apoptosis 2005; **10**: 731–741

The release of microparticles by apoptotic cells and their effects on macrophages

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Microparticles are small membrane vesicles released from the cell membrane by exogenous budding. To elucidate the interactions of microparticles with macrophages, the effect of microparticles released from Jurkat T cells on RAW 264.7 cells was determined. Microparticles were isolated by differential centrifugation, using FACS analysis with annexin V and cell surface markers for identification. Various inducers of apoptosis increased the release of microparticles from Jurkat cells up to 5-fold. The released microparticles were then cultured with RAW 264.7 cells. As shown by confocal microscopy and FACS analysis, RAW 264.7 macrophages cleared microparticles by phagocytosis. In addition, microparticles induced apoptosis in RAW 264.7 cells in a dose-dependent manner with up to a 5-fold increase of annexin V positive cells and 9-fold increase in caspase 3 activity. Cell proliferation as determined by the MTT test was also reduced. Furthermore, microparticles stimulated the release of microparticles from macrophages. These effects were specific for macrophages, since no apoptosis was observed in NIH 3T3 and L929 cells. These findings indicate that microparticles can induce macrophages to undergo apoptosis, in turn resulting in a further increase of microparticles. The release of microparticles from apoptotic cells may therefore represent a novel amplification loop of cell death.

Keywords: apoptosis; cell death; microparticles.

Introduction

Cell death is an integral cellular process that can occur by two major mechanisms. Apoptosis, or programmed cell death, is an active, energy dependent process which causes typical morphological changes, including cell shrinkage, nuclear condensation and fragmentation, and membrane alterations.[1] Apoptosis usually affects scattered cells in a tissue and is triggered by a host of stimuli in both physiologic and pathologic settings. In contrast, necrosis, or accidental cell death, is a pathologic process induced by physical or chemical stimuli and is characterized by cellular disintegration and the release of toxic components.[1]

In addition to morphological and biochemical changes, cells dying by apoptosis and necrosis differ in their immunological effects. Whereas apoptotic cells are anti-inflammatory, necrotic cells are pro-inflammatory.[2–6] These activities reflect membrane changes, especially the exposure of phosphatidylserine on the membrane, as well as the release of intracellular molecules such as the high mobility group B1 protein.[7] The extent to which these activities are manifest, however, depends upon cell clearance since both apoptotic and necrotic cells are readily phagocytized.[8–10] This process is mediated primarily by macrophages, which recognize membrane changes on dead and dying cells and promote their phagocytosis and engulfment for safe disposal.[11] In addition to professional phagocytes, non-professional phagocytes such as neighboring parenchymal cells can also engulf dead and dying cells. The rapid removal of dead cells may ensure tissue homeostasis as well as modulate local inflammation.[11,12]

During the death process, cells undergo extensive macromolecule changes that encompass cleavage and translocation. While the function of these changes is not fully known, they may provide a mechanism for the safe removal of cellular debris, including signaling for uptake by phagocytes as well as elimination, modification or detoxification of certain cellular constituents. Among these changes is the release of microparticles. These particles, which can be defined by size and sedimentation properties, represent a heterogeneous population of vesicles released from the cell membrane by exocytic budding.[13] The normal membrane asymmetry is lost during the budding process and phosphatidylserine and other aminophospholipids appear on the outer leaflet of the microparticle membrane. In addition to cell surface markers such as MHC

Support for this study came from a VA Merit Review grant and NIH grant AI44808
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class II and costimulatory molecules, microparticles carry proteins derived from the cytosol, the endoplasmatic reticulum and the nucleus, including histones.[14] Release of microparticles has been observed from virtually all cell types, with inducers of apoptosis as well as agents like LPS or proinflammatory cytokines causing their release.[15,16]

Given their array of cell surface molecules, microparticles may represent another component of apoptotic cells that can influence host responses, including induction of thrombosis as well as inflammation. Secreted microparticles can act as procoagulant surfaces by exposing negatively charged phospholipids such as phosphatidylserine.[17,18] Microparticles may also bear tissue factor, contributing to the prothrombotic activity.[17,19,20] Furthermore, depending on their source, microparticles can induce or modulate immune responses, contributing to disease states such as atherosclerosis, multiple sclerosis and rheumatoid arthritis.[21,22] In the current studies, we have investigated the relationship between apoptosis and the generation of microparticles. In addition, we have assessed the interaction of microparticles with macrophages. Results of these studies indicate that microparticles derived from Jurkat cells can induce apoptosis dose-dependently in the RAW 264.7 murine macrophage cell line. In turn, RAW 264.7 release microparticles themselves. Together, these results suggest that constituents of apoptotic cells, as well as the cells themselves, have immunological activities, thus extending the range of downstream effects of apoptosis on the organism.

Material and methods

Cell culture and induction of apoptosis

Mouse RAW 264.7 macrophages, mouse NIH 3T3 fibroblasts, mouse L929 fibrosarcoma cells and human Jurkat T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Carlsbad, CA) containing 10% heat inactivated fetal bovine serum (FBS) (Hyclone, Logan UT) and 20 μg/ml Gentamicin (Gibco). For the induction of apoptosis, 10^8 Jurkat cells were stimulated with either etoposide (10 μM), actinomycin-D (5 μg/ml), TNFα (100 μM) or staurosporine (10 μM) (all Sigma Chemical Co., St Louis, MO) for 24 h or irradiation with a UVA light source (UVP, San Gabriel, CA) for 10 min at a distance of 10 cm. Alternatively, Jurkat cells were heated at 56°C for 30 min in a water bath. Afterwards, the supernatants were collected and the microparticles were isolated immediately. For coculture experiments, 2×10^6 confluent RAW 264.7 cells were incubated for 36 h with freshly isolated microparticles from 2×10^5 to 2×10^7 apoptotic Jurkat cells. RAW 264.7 cells cultured without Jurkat microparticles were used as controls.

Isolation of microparticles by differential centrifugation

Cell culture supernatants were centrifuged at 1500 $\times$ g for 5 min to remove suspended cells. Afterwards, the cell-free supernatants were centrifuged at 100000 $\times$ g at 20°C for 20 min. The supernatant was removed and the pellet was washed twice with 10 ml apop buffer (5 mM KCl, 1 mM $MgCl_2$ and 136 mM NaCl, pH 7.4) and finally resuspended in 2 ml apop buffer.[21,23] The microparticles were then either quantified and characterized by flow cytometry analysis or used for coculture experiments with RAW 264.7, NIH 3T3 or L929 cells. The number of microparticles as counted by FACS was adjusted to the number of RAW 264.7 cells. RAW 264.7 macrophages were counted with a hemocytometer and 2×10^6 cells in 5 ml culture medium were used for the experiments.

Flow cytometry analysis (FACS)

For characterization and quantification of microparticles by FACS, freshly isolated microparticles were diluted in apop buffer with 2.5 mM $CaCl_2$ and 1% microparticle-free FBS to a final volume of 200 μl. Microparticles were preincubated with 5 μl monoclonal anti-human CD3 R-phycoerythrin (RPE) (Clone UCHT, Sigma) or 50 μl monoclonal anti-mouse F4/80 RPE (Clone Cl:AS-1, Serotec, Raleigh, NC) for 30 min at room temperature in the dark. Double staining was performed with 5 μl annexin V-FITC (BD Pharmingen, San Diego, CA) for additional 30 min. Unbound antibodies and annexin V were removed by two washing steps at 100000 $\times$ g for 20 min. Staining with isotype antibodies and annexin V in the absence of calcium was used as controls. After the last washing step, microparticles were resuspended in 1 ml apop buffer with 2.5 mM $CaCl_2$ and 1% microparticle-free FBS. The number of microparticles was determined by measuring 1 min at the "hi-flow" modus and calculation of the total amount of microparticles by multiplication with the ratio of total volume to measured volume.

To study the effects of microparticles on macrophages, subconfluent RAW 264.7 cells were co-cultured with increasing amounts of microparticles derived from Jurkat cells. After 36 h, microparticles in the supernatant were removed by two washing steps and RAW 264.7 cells were harvested. Cells incubated with apop buffer in the absence of Jurkat microparticles were used as controls. For the assessment of cell viability by FACS, cells were resuspended in 200 μl PBS/ 1% FBS containing 5 μl annexin V-FITC and 10 μl of a 50 μg/ml propidium iodide solution (PI) (Sigma), incubated for 30 min at room temperatur and then washed with PBS/1%FBS. The analysis was performed using the FACScan flow cytometer (Becton

Dickinson, Mansfield, MA) and the data were evaluated with CellQuest software (Becton Dickinson Immunocytometry System, San Jose, CA).

Although microparticles and apoptotic cells were both positive for annexin V, they could be easily distinguished by FACS by forward scatter because of their differences in size.

Staining of microparticles and fluorescence microscopy

Microparticles were isolated freshly as described above and membranes labeled with the fluorescent membrane cell linker PKH26 (Sigma) using the manufacturer's protocol.

For conventional fluorescence microscopy, stained microparticles were coincubated with RAW 264.7 cells in 8 well chambered coverslips (Nalge Nunc, Naperville IL) for 12 h. Microscopy was performed and images captured with an Olympus I × 51 inverted microscope equipped with an Olympus DP70 digital camera (Olympus Optical, Tokyo).

For confocal fluorescence microscopy, 3×10^6 stained microparticles were added to RAW 264.7 cells grown in 24 well plates containing round coverslips (Hecht Assistant GmbH, Altnau, Switzerland). For inhibition of phagocytosis, 20 μM cytochalasin D (Fluka, Buchs, Switzerland) was added in a subset of experiments. After incubation at 4°C for 30 minutes and at 37°C for another 30 minutes, RAW 267.4 cells were fixed with paraformaldehyde and the nuclei were stained with DAPI. Confocal fluorescence microscopy was performed using the Leica SP2 inverted microscope (Leica, Wetzlar, Germany).

Caspase 3 activity assay

RAW 264.7 cells were cocultured with various amounts of microparticles as described above. The activities of caspase-3-like proteases were determined using the EnzChek caspase-3 assay kit (Molecular Probes). This assay is based on the cleavage of the non-fluorescent rhodamine-110 bis-*N*-CBZ-L-aspartyl-L-gluamyl-L-valyl-L-aspartic acid amine (Z-DEVD-R110) into the fluorescent rhodamine-100 by caspase-3-like proteases. Fluorescence was measured in a TECAN GENios microplate fluorescence reader (Tecan, Salzburg, Austria) at an excitation wavelength of 485 nm and emission of 535 nm. Background fluorescence was determined by measuring substrate cleavage in the presence of the caspase-3 inhibitor Ac-DEVD-CHO. Results are expressed as arbitrary fluorescence units (AFU).

Microtiter tetrazolium (MTT) assay

RAW 264.7 macrophages were cocultured with microparticles in 96 well plates for 32 h. After removing 100 μl of the growth medium, MTT was added at a final concentration of 1 mg/ml and the cells were incubated at 37°C for additional 4 h. After dilution with 100 μl 0.04 N HCl in isopropanol, the wells were analyzed using an automatic ELISA plate reader (UV MAX, Molecular Devices, Sunnyvale, CA) at a test wavelength of 570 nm. The number of microparticles was adjusted to the reduced cell number (1.3×10^4 RAW 264.7 cells) used for the MTT assay to yield the same ratio of RAW 264.7 cells to microparticles as in the previous experiments. The proliferation capacity of RAW 264.7 cells that were not cocultured with microparticles was defined as 100%; other results are shown as relative to this value.

Statistical analysis

Data are expressed as mean ± standard deviation. The Wilcoxon signed rank test for related samples and the Mann-Whitney test for non-related samples were used for statistical analyses. A p-value of less than 0.05 was considered statistically significant.

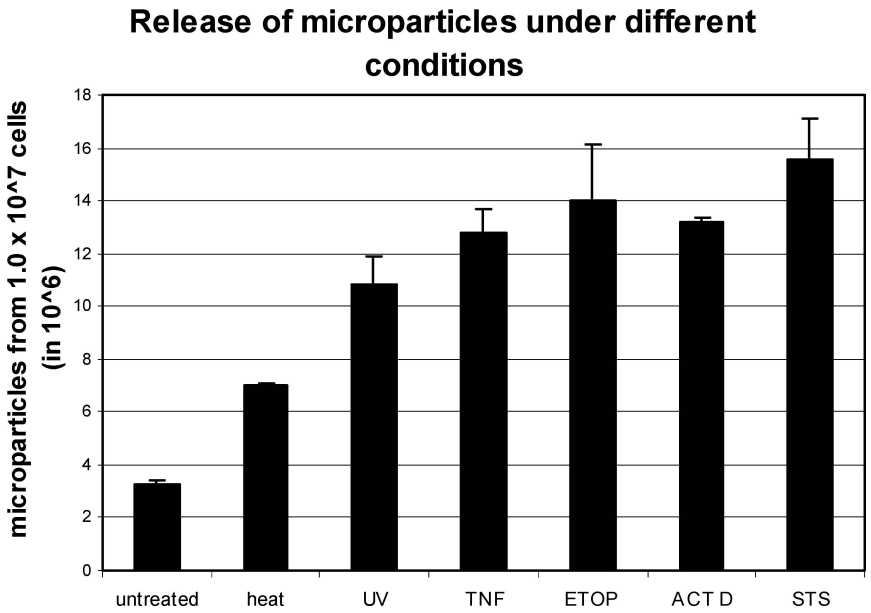
Results

Quantification of microparticles released from Jurkat cells

The production of microparticles was first tested in Jurkat cells in the basal state as well as following treatments to induce apoptosis. Microparticles were isolated by differential centrifugation, using FACS analysis for annexin V and CD3, a marker for T cells, for identification and quantification. Using this method, we found that the culture medium of 10^8 untreated Jurkat cells contained $3.29 \times 10^6 \pm 1.53 \times 10^5$ microparticles under the culture conditions used. The number of microparticles increased to $6.98 \times 10^6 \pm 1.35 \times 10^5$ in heat-treated Jurkat cells (2.1 fold increase, $p \leq 0.05$).

Induction of apoptosis by UV-irradiation or chemical compounds such as etoposide, actinomycin D or staurosporine resulted in a larger amount of released microparticles. After UV irradiation, 10^8 Jurkat cells released $1.08 \times 10^7 \pm 1.03 \times 10^6$ microparticles (3.3 fold increase, $p \leq 0.03$). Etoposide and actinomycin D increased the release of microparticles by 4.4 and 4.0 fold, with the release of $1.40 \times 10^7 \pm 2.12 \times 10^6 (p \leq 0.01)$ and $1.32 \times 10^7 \pm 1.87 \times 10^5 (p \leq 0.01)$ microparticles, respectively. The highest amount of microparticles was observed after stimulation with staurosporine

Figure 1. Release of microparticles from Jurkat T cells. Apoptosis was induced either by UV-irradiation or by incubation with etoposide, actinomycin D or staurosporine. Untreated: basal release of microparticles from Jurkat cells; UV: UV irradiated Jurkat cells; ETOP: etoposide treated cells; ACT D: actinomycin D treated cells; STS: staurosporine treated cells.



($1.56 \times 10^7 \pm 1.53 \times 10^6$), corresponding to a 4.8 fold induction compared to unstimulated controls ($p \leq 0.01$) (Figure 1).

RAW 264.7 cells can clear microparticles by phagocytosis

Macrophages have been shown to remove apoptotic cells with high efficiency.[4,28] To investigate the potential of macrophages to remove microparticles, RAW 264.7 cells were incubated with PHK 26 stained microparticles and the disposition and localization of the microparticles was analyzed by fluorescence microscopy. After 12 h, most of the stained microparticles were bound to, or incorporated by, RAW 264.7 macrophages; only a small fraction was found to be extracellular after incubation.

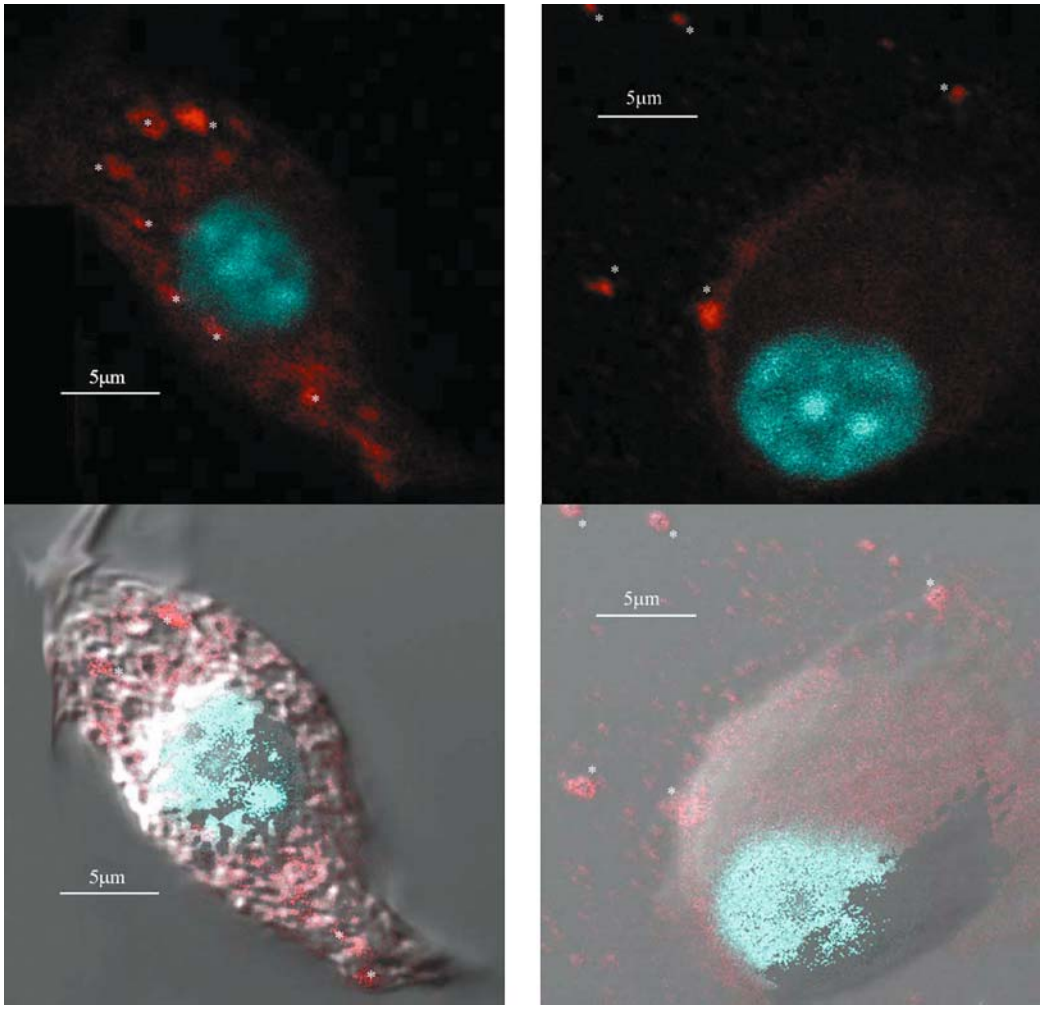
To distinguish whether microparticles were attached to the cell surface or incorportated into RAW 264.7 macrophages, confocal microscopy was performed. As shown in Figures 2a and c, after 60 minutes the stained microparticles were found incorporated by RAW 264.7 macrophages. The macrophages taking up microparticles contained microparticles within the cytoplasm, whereas the nuclei did not display staining from particles (Figure 2a). However, when RAW 264.7 cells were incubated with 20 μM Cytochalasin D immediately before adding the stained microparticles to inhibit phagocytosis, the fluorescent signals from microparticles were exclusively localized in the extracellular space around the macrophages (Figure 2b).

The ability of RAW 264.7 cells to phagocytose microparticles was further evaluated by FACS. In these experiments, increasing concentrations of microparticles were added to RAW 264.7 cells and the number of non-phagocytosed CD3/annexin positive microparticles derived from Jurkat cells was analyzed by FACS. As shown in Figure 3. 3×10^4 were removed efficiently by 2.0×10^6 RAW 264.7 cells, with almost no CD3 positive microparticles detectable in the supernatant after 36 h of coculture. With 3.0×10^5 microparticles added to RAW 264.7 cells, however, microparticles from the Jurkat cells were still detectable after 36 h of co-culture. Further increases in the amount of microparticles up to 3.0×10^6 microparticles resulted in a progressive accumulation of CD3 positive microparticles (Figure 3). No significant differences were found between microparticles of different origin.

Microparticles reduce the incorporation of MTT into RAW 264.7 macrophages

Microscopic examination of RAW 264.7 cells after incubation with different amounts of microparticles showed a lower cell density. To confirm this finding, the incorporation of MTT into RAW 264.7 cells after incubation with microparticles was meassured. No significant differences in the cleavage of MTT were observed after incubation with low amounts of microparticles (2×10^2 to 2×10^3 microparticles) compared to controls (Figure 4). The addition of 2×10^4 microparticles, however, markedly decreased the intake of MTT into RAW 264.7

Figure 2. Microparticles are incorporated into RAW 264.7 cells. (a): Phagocytosis of microparticles by RAW 264.7 cells assessed with confocal fluorescence microscopy. Nuclei of RAW 264.7 cells stained with DAPI (blue signals), microparticles stained with the membrane marker PKH26 (red signals). Microparticles (red signals) are localized in the cytoplasm of the RAW 264.7 macrophages, whereas no microparticles were found in the nucleus. (b): Inhibition of phagocytosis by Cytochalasin D prevents the uptake of microparticles (red signals) by RAW 264.7 macrophages. Overlay of DAPI (nucleus stain) and PKH26 (microparticles) in the upper panels, additional bright field picture acquired by differential interference contrast (dit) in the lower panels, respectively. Microparticles are indicated by white stars.



cells. Microparticles from UV-irradiated Jurkat cells decreased the proliferation to 75.3 ± 17.0% ($p \leq 0.05$), respectively. Microparticles derived from apoptotic Jurkat cells treated with etoposide, actinomycin D and staurosporine resulted in a strong reduction of RAW 264.7 cells to 37.6 ± 1.4%($p \leq 0.01$), 30.6 ± 3.6%($p \leq 0.01$) and 29.0 ± 13.9% ($p \leq 0.01$) compared to controls.

Microparticles induce dose-dependently apoptosis in RAW 264.7 macrophages

The lower numbers of cells and the reduced incorporation of MTT into RAW 264.7 cells incubated with microparticles might be either due to decreased proliferation or to increased cell death. To measure directly the extent of apoptosis in RAW 264.7 cultures incubated with increasing numbers of microparticles, cells were stained with annexin V and propidium iodide (PI) and analyzed by FACS. In these experiments, controls without microparticles showed 3.7 ± 1.3% cells positive for annexin V only and 2.6 ± 0.92% positive cells for both annexin V and PI with a total amount of annexin V positive cells of 6.3 ± 2.3%.

The percentage of RAW 264.7 cells that were positive only for annexin V as well as the number of annexin/PI double positive cells and the total amount of annexin positive microparticles correlated with the number of microparticles added (Figure 5). With lower doses of 3×10^4 microparticles, there was no significant increase in the percentage of annexin V positive RAW 264.7 cells compared

Figure 3. Clearance of Jurkat-derived microparticles by RAW 264.7 macrophages. The phagocytosis by RAW 264.7 cells of microparticles was assessed in coculture experiments of RAW 264.7 cells with increasing amounts of microparticles. Lower amounts of microparticles were cleared efficiently with almost none detectable after 36 h. The clearance capacity of RAW 264.7 cells was exceeded, when 3×10^6 microparticles were added, with up to $2.3 \times 10^5 \pm 1.6 \times 10^4$ CD 3 positive microparticles detectable after 36 h. UV: UV irradiated Jurkat cells; ETOP: etoposide treated cells; ACT D: actinomycin D treated cells; STS: staurosporine treated cells; MPs: microparticles.

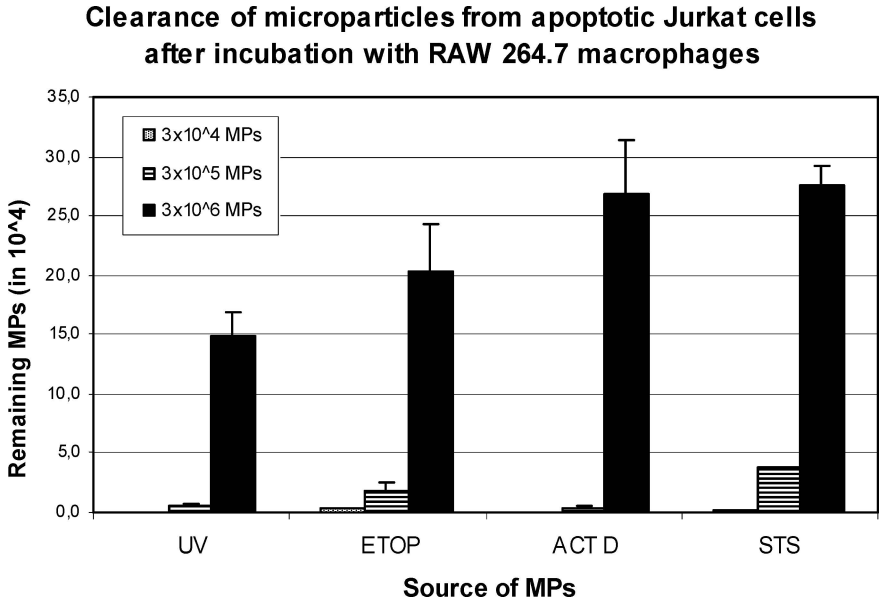


Figure 4. Reduced incorporation of MTT into RAW 264.7 cells after coculture with microparticles. The proliferation of untreated RAW 264.7 cells was defined as 100% and all other results were normalized to this value. With 2×10^2 to 2×10^3 microparticles, no significant differences were detected in the cleavage of MTT compared to controls. In contrast, 2×10^4 microparticles reduced to proliferation rate of Raw 264.7 macrophages, with maximal decreases to $29.0 \pm 13.9\%$. UV: UV irradiated Jurkat cells; ETOP: etoposide treated cells; ACT D: actinomycin D treated cells; STS: staurosporine treated cells; MPs: microparticles.

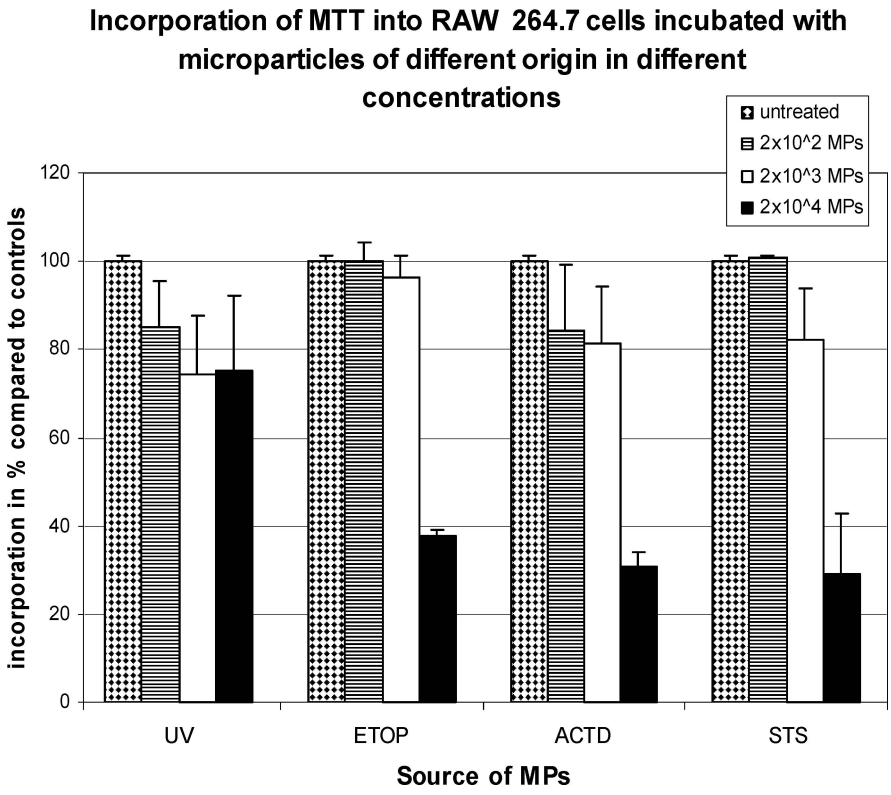
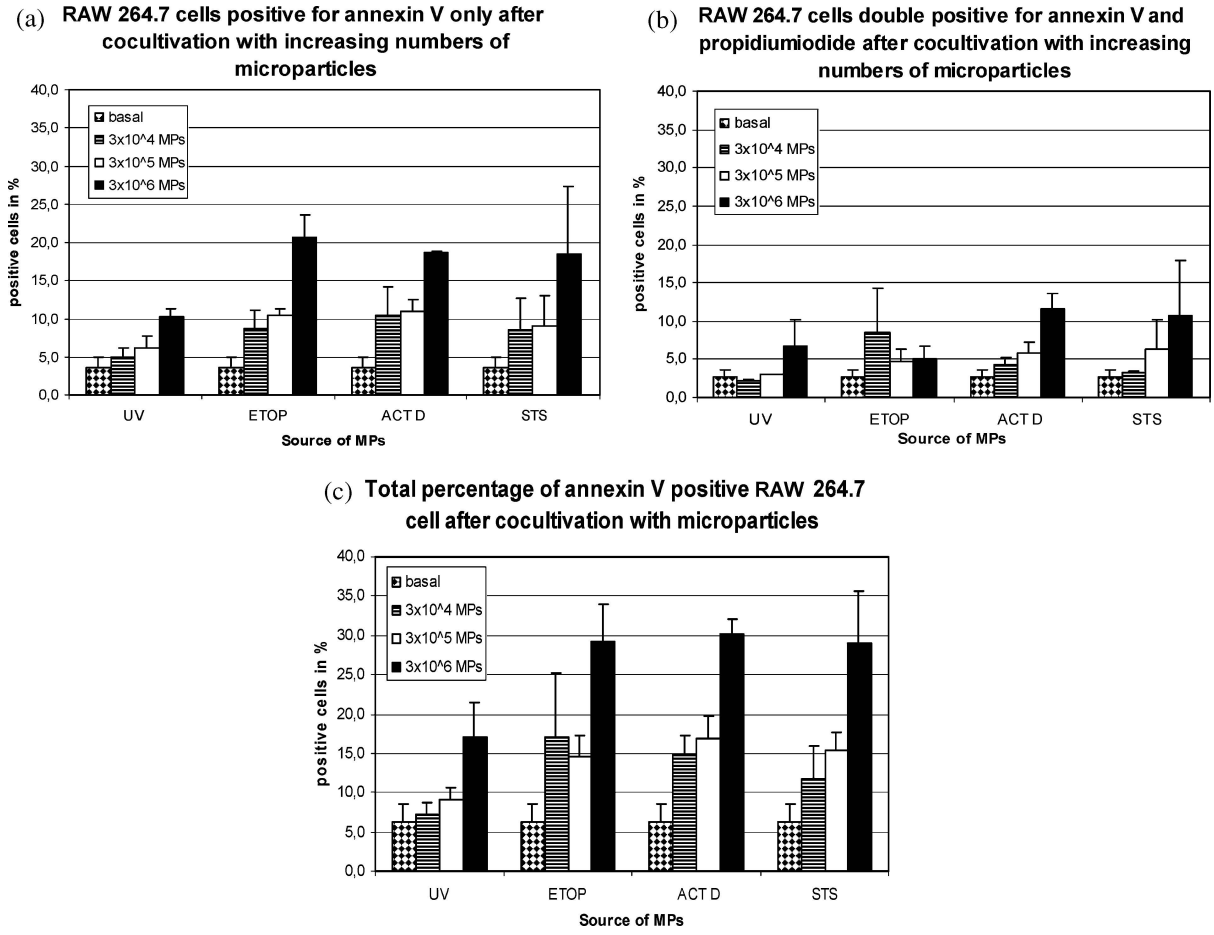


Figure 5. Dose-dependent induction of apoptosis in RAW 264.7 macrophages by microparticles. No significant differences were observed between controls and RAW 264.7 cells incubated with up to 3×10^4 microparticles. However, dose dependent increases in the percentage of RAW 264.7 cells positive for annexin V was observed with 3×10^5 to 3×10^6 microparticles with up to $29.2 \pm 6.5\%$ annexin V positive cells. (a): RAW 264.7 cells positive only for annexin V, but not for PI, suggesting an early stage of apoptosis. (b): RAW 264.7 cells double positive for annexin V and PI, suggesting a later stage of apoptosis. (c): Total percentage of annexin positive, apoptotic RAW 264.7 cells. UV: UV irradiated Jurkat cells; ETOP: etoposide treated cells; ACT D: actinomycin D treated cells; STS: staurosporine treated cells; MPs: microparticles.

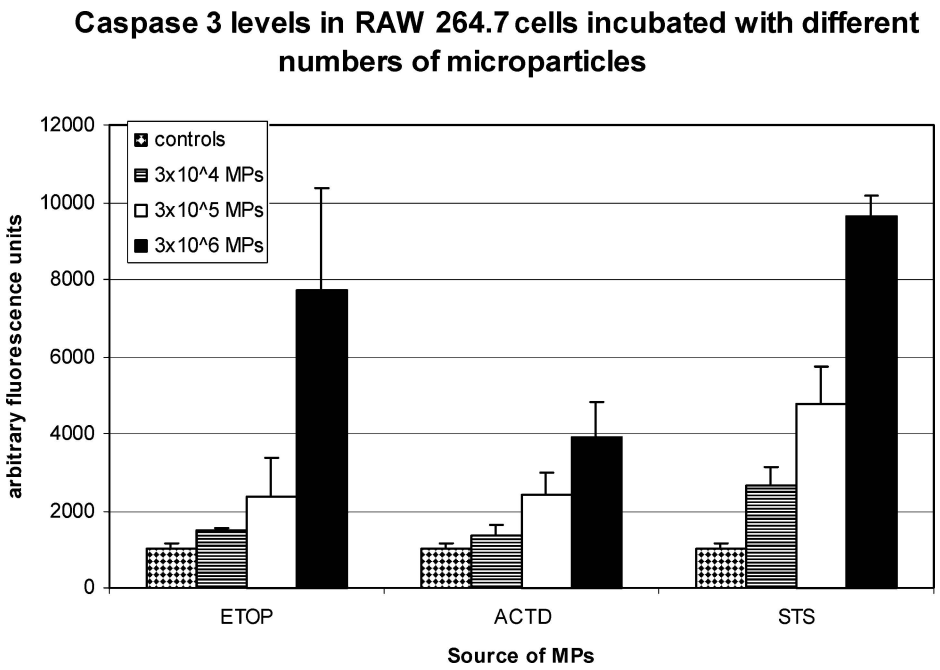


to controls. Incubation with 3×10^5 to 3×10^6 microparticles, however, resulted in a dose-dependent increase of apoptotic macrophages. After incubation with 3×10^5 microparticles from Jurkat cells treated with staurosporine, $9.1 \pm 3.9\%$ of the RAW 264.7 cells were positive for annexin V only ($p \leq 0.03$), while $6.3 \pm 3.8\%$ were double positive for annexin V and PI ($p \leq 0.03$). A further increase of the number of microparticles to 3.0×10^6 augmented the total number of annexin positive cells to $29.1 \pm 3.8\%$ ($p \leq 0.03$), with $18.4 \pm 8.8\%$ of the cells positive for annexin V only ($p \leq 0.01$) and $10.6 \pm 7.3\%$ annexin V / PI double positive RAW 264.7 cells ($p \leq 0.03$) (Figure 5). Similar results were obtained with microparticles from apoptotic Jurkat cells treated with UV-irradiation, etoposide or actinomycin D. Together, these results suggest that microparticles can induce macrophage apoptosis.

Upregulation of caspase 3 in RAW 264.7 cells by microparticles

To confirm the induction of macrophage apoptosis with a second independent method, caspase 3 levels in RAW 264.7 cells were measured after incubation with different amounts of microparticles. Controls showed a mean caspase 3 activity of 1021 ± 117 AFU. No significant increase in caspase 3 activity was detected when RAW 264.7 cells were stimulated with 3×10^4 microparticles ($p \leq 0.01$) with the exception of microparticles derived from Jurkat cells stimulated with staurosporine (2988 ± 481, $p \leq 0.05$). Consistent with the results from the annexin V / PI staining, addition of greater amounts of microparticles (3×10^5 to 3×10^6 microparticles) increased the activity of caspase 3 significantly compared to controls and lower doses of microparticles

Figure 6. Dose-dependent increase of caspase 3 activity in RAW 264.7 cells by microparticles. Higher concentrations of microparticles from apoptotic Jurkat cells upregulated the activity of caspase 3 significantly with a maximum increase of 9.4 fold compared to controls. ETOP: etoposide treated cells; ACT D: actinomycin D treated cells; STS: staurosporine treated cells; MPs: microparticles.



(Figure 6). The activity of caspase 3 was upregulated to 4776 ± 943 AFU after coculture with 3×10^5 microparticles from staurosporine-treated Jurkat cells (4.7 fold increase compared to controls, $p \leq 0.03$). After addition of 3×10^6 microparticles, a mean caspase 3 activity of 9661 ± 492 AFU (9.1 fold increase, $p \leq 0.03$) was measured in RAW 264.7 cells. Similar results were obtained with microparticles derived from Jurkat cells with other stimuli of apoptosis (Figure 6).

The induction of apoptosis by microparticles is specific for macrophages

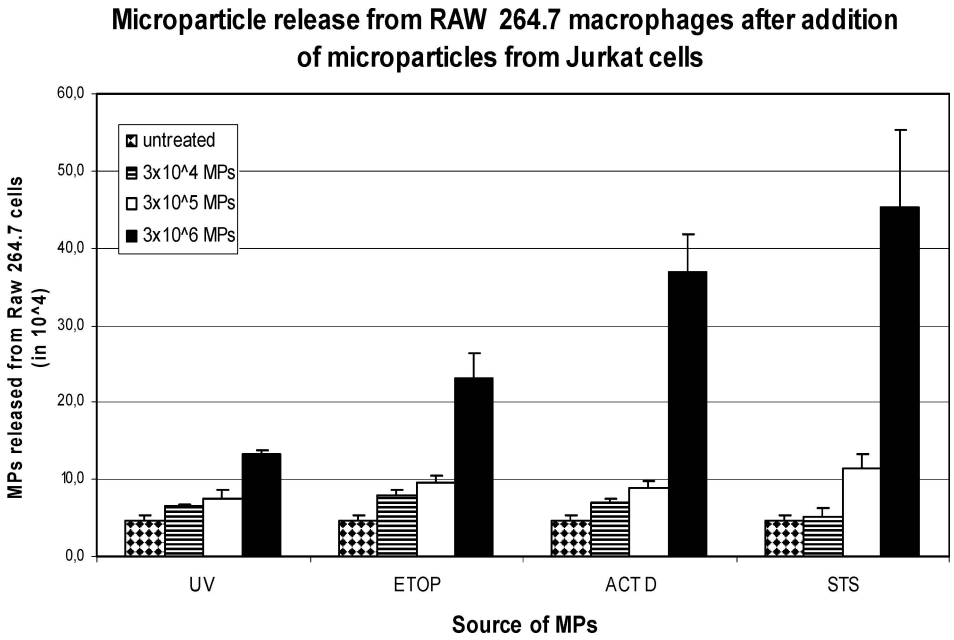
Macrophages are specialized cells that are highly phagocytic. Other cell types such as fibroblasts can also ingest foreign materials,[24] but their phagocytic capacity is limited compared to macrophages. To investigate whether the induction of apoptosis by microparticles is a general feature of cells exposed to microparticles or is specific for macrophages, NIH 3T3 fibroblasts and L929 fibrosarcoma cells were cocultured with microparticles and the percentage of annexin V positive cells, the activity of caspase 3 and the proliferation rate were measured. In contrast to results with RAW 264.7 macrophages, no evidence of increased apoptosis was detected with any of these assays after incubation with microparticles from apoptotic Jurkat cells in various concentrations. These results were found for both 3T3 NIH as well as L929 cells (data not shown).

Stimulation of microparticle release from RAW 264.7 cells by Jurkat microparticles

As these data suggest, microparticles can induce apoptosis in RAW 264.7 cells. Since apoptotic cells can release microparticles, we therefore tested whether microparticles from Jurkat cells can induce the release of microparticles from RAW 264.7 cells. For this purpose, we assessed the induction of microparticles from macrophages by staining with anti-mouse F4/80 RPE antibodies. Preexperiments demonstrated that there was no unspecific staining of microparticles derived from Jurkat cells with anti-F4/80 RPE antibodies.

Similar to Jurkat cells, RAW 264.7 cells showed a low basal release of 4.59×10^4 microparticles. Consistent with the results of the previous experiments, no significant changes were observed after coculture with 3×10^4 Jurkat microparticles. In contrast, a significant increase of RAW 264.7-derived, F4/80 antigen positive microparticles was detectable after addition of 3×10^5 Jurkat microparticles. With further increases in the amounts of microparticles to 3×10^6 microparticles present in the co-cultures, the release of microparticles from RAW 264.7 cells was enhanced in a dose-dependent manner (Figure 7). 3×10^5 microparticles from apoptotic Jurkat cells treated with staurosporine induced a 2.5 fold increase of the microparticle release ($1.1 \times 10^5 \pm 1.9 \times 10^4$, $p \leq 0.03$). After cocultivation with 3×10^6 microparticles, 9.9 fold more microparticles ($4.5 \times 10^5 \pm 1.0 \times 10^5$, $p \leq 0.03$)

Figure 7. Dose-dependent increase in the release of macrophage specific microparticles after coculture with microparticles from Jurkat cells. The highest doses of microparticles (3×10^6 microparticles) induced increases in the numbers of macrophage-microparticles of up to 9.9 fold compared to controls. UV: UV irradiated Jurkat cells; ETOP: etoposide treated cells; ACT D: actinomycin D treated cells; STS: staurosporine treated cells; MPs: microparticles.



from RAW 264.7 cells were detectable than in controls. Similar effects were observed with microparticles from UV irradiated and etoposide or actinomycin D treated Jurkat cells. Together, these observations indicate that microparticles can induce the generation of microparticles from macrophages, with apoptosis the likely mechanism.

Discussion

The induction of macrophage apoptosis by microparticles is unexpected since macrophages have been viewed as a key scavenger for dead and dying cells and essential for the clearance of cellular debris from the organism.[8] Indeed, the high capacity of macrophages to engulf dead and dying cells may explain the difficulty in identifying apoptotic cells in tissue. In our experiments, the induction of apoptosis by microparticles appeared to depend on internalization, since increased apoptosis was observed only in RAW 264.7 cells with a high phagocytic capacity, but not in NIH 3T3 and L929 cells with limited or no ability to engulf cells. Together, these findings suggest that, following uptake, ingested particles at a critical intracellular concentration can activate the apoptosis program.[25–28] At present, the molecules responsible for the induction of apoptosis are speculative. They could either be molecules that have been degraded, cleaved or otherwise modified during cell death. Alternatively, they could represent an intact molecule that is able to induce apoptosis because it is concentrated in the microparticles and can enter a subcellular compartment following phagocytosis.

In previous studies investigating the mechanisms for the clearance of apoptotic and necrotic cells *in vivo*, we demonstrated that the administration of dead and dying Jurkat cells to normal mice leads to the appearance of DNA of both human and murine origin in the blood.[29] Furthermore, this DNA showed a laddering pattern consistent with DNA cleavage during apoptosis. This release, however, was dependent on the presence of macrophages since mice in which macrophages were eliminated or functionally inactivated by treatment with clodronate liposomes or silica failed to produce a blood DNA response. To explain these findings, we suggested that the release of DNA in this system depends at least in part on the induction of the apoptotic pathway in macrophages that have engulfed excessive numbers of dead and dying cells. In this situation, the DNA of the engulfed cells is cleaved, with release occurring as the macrophage dies, releasing its DNA as well. *In vitro* studies support this possibility.[29] On the basis of these observations, we would propose that the uptake of microparticles by macrophages induces apoptosis in a dose-dependent process, with the release of microparticles from macrophages that have engulfed the Jurkat microparticles similar to the release of DNA from macrophages that have engulfed apoptotic or necrotic Jurkat cells.

While the mechanisms by which microparticles induce apoptosis require further investigation, these studies have clinical implications. Massive apoptosis occurs in a variety

of settings such as treatment of malignancy by chemotherapy or radiation or tumor lysis syndromes[30,31] that cause the release of large amounts of microparticles. Since these microparticles have the potential to induce apoptosis in macrophages, the result could be both immune suppression and a self-increasing amplification of microparticles. With the loss of macrophages, the clearance of apoptotic cells might be impaired and apoptotic cells could undergo secondary necrosis. This situation could intensify inflammation and injury, contributing to the poor prognosis observed in these conditions.

Studies with both *in vitro* and *in vivo* systems have demonstrated that the uptake of dead and dying cells by macrophages is highly efficient.[11,28,32] In contrast to the uptake of necrotic cells, the uptake of apoptotic cells is thought to suppress immune responses and to cause an anti-inflammatory state.[2–6] Macrophages, however, can be activated to secrete pro-inflammatory mediators by the ingestion of secondary necrotic cells. Therefore, apoptotic cells can trigger inflammatory responses, if they are not cleared properly and are allowed to undergo secondary necrosis.

The incomplete clearance of apoptotic cells can occur in two distinct settings. Thus, dying cells can accumulate in either patients with defects in the phagocytosis of apoptotic cells or during massive cell death, when large numbers of dying cells exceed the clearance capacity of the organism. Indeed, defects of phagocytosis of apoptotic cells have been linked closely to autoimmune and chronic inflammatory diseases.[33] Under normal circumstances, C1q binds to defined binding sites on the surface of apoptotic cells. This complex is then recognized by C1q receptors on the surface of phagocytes and promotes the engulfment of the apoptotic cell.[34]

Interestingly, patients deficient in C1q suffer almost invariably from a SLE-like illness and $C1q^{-/-}$ mice spontaneously develop features of autoimmunity with high levels of non-ingested apoptotic cells. The administration of exogenous C1q can prevent the autoimmune features. In our studies, we demonstrated the ability of microparticles to induce apoptosis in RAW 264.7 macrophages. Microparticles released upon cell death might not only claim a part of the clearance capacity for apoptotic cells, but also might reduce the total capacity by diminishing the number of viable macrophages. This effect could lead to increased numbers of cells undergoing secondary necrosis, causing a proinflammatory phenotype similar to that observed with deficiency in phagocytosis.

The number of microparticles is increased in various autoimmune diseases such as rheumatoid arthritis,[35] vasculitis[36] and antiphospholipid syndrome.[17] Interestingly, leukocyte microparticles have been demonstrated to stimulate the release of proinflammatory cytokines such as interleukin-6, interleukin-8 and members of the family of monocyte-chemoattractant proteins from various cell types.[15,37] In addition, ICAM, E-selectin and VCAM-1 are induced in endothelial cells, thereby facilitating leukocyte adhesion and extravasation.[15] These findings suggest that, in addition to their effects on macrophages, microparticles from leukocytes might contribute directly to the persistence of inflammation. Together, these findings point to microparticles as novel signaling elements of disease that can potentiate inflammation and autoimmunity at a variety of steps in pathogenesis.

Conclusion

The studies presented herein clarify the manner in which apoptotic cells release their contents and the effect of these contents on other cells. Thus, we showed that apoptotic Jurkat cells in culture release microparticles. In the presence of RAW 264.7 macrophages, these microparticles were engulfed, with high levels of microparticles causing apoptosis in the macrophage cell line, but not in other cell types. The ingestion of microparticles might therefore represent a novel and specific mechanism for the induction of apoptosis in macrophages. Furthermore, the induction of macrophage apoptosis was associated with the release of microparticles from apoptotic macrophages. In situations where excessive apoptosis occurs, e.g. chemotherapy of hematologic neoplasias, microparticles might therefore trigger an amplification loop of apoptosis and release of microparticles. Microparticles could also modify immune responses to apoptotic cells by inducing loss of macrophages. In the absence of macrophages, clearance of apoptotic cells might be insufficient and apoptotic cells might undergo secondary necrosis, thereby triggering inflammation instead of suppressing it.

Acknowledgments

The authors thank all staff members of the laboratory of electron microscopy (EMZ) at the University of Zurich for support, in particular Dr. M. Hoechli for assistance at the confocal microscope, and B. Knecht for fixation and staining procedures.

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