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Distinct Signaling Pathways Are Involved in Leukosialin (CD43) Down-regulation, Membrane Blebbing, and Phospholipid Scrambling during Neutrophil Apoptosis*[§]

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Although leukosialin (CD43) membrane expression decreases during neutrophil apoptosis, the CD43 molecule, unexpectedly, is neither proteolyzed nor internalized. We thus wondered whether it could be shed on bleb-derived membrane vesicles. Membrane blebbing is a transient event, hardly appreciated during the asynchronous, spontaneous apoptosis of neutrophils. Cell pre-synchronization at 15 °C made it possible to observe numerous blebbing neutrophils for a short 1-h period at 37 °C. CD43 down-regulation co-occurred with the blebbing stage and phosphatidylserine externalization, shortly after mitochondria depolarization and before nuclear condensation. Blebs detaching from the cell body were observed by time lapse fluorescence microscopy, and the release of bleb-derived vesicles was followed by flow cytometry. Phosphatidylserine externalization required caspases and protein kinase C (PKC) but not the myosin light chain kinase (MLCK). By contrast, bleb formation and release was caspase- and PKC-independent but required an active MLCK, whereas CD43 down-regulation involved caspases but neither PKC nor MLCK. Furthermore, CD43 appeared mostly excluded from membrane blebs by electron microscopy. Thus, CD43 down-regulation does not result from the release of bleb-derived vesicles. Ultracentrifugation of apoptotic cell supernatants made it possible to recover <1 µm microparticles, which contained the entire CD43 molecule. These microparticles expressed neutrophil membrane markers such as CD11b, CD66b, and CD63, together with CD43. In conclusion, we show that the three early membrane events of apoptosis, namely blebbing, phosphatidylserine externalization, and CD43 down-regulation, result from different signaling pathways and can occur independently from one another. CD43 down-regulation results from the shedding of microparticles released during apoptosis but unrelated to the blebbing.

ments result in impressive membrane protrusions, described as membrane “budding” or “blebbing” (1). (ii) Phospholipids, such as phosphatidylserine (PS),¹ are relocated from the inner to the outer leaflet of the membrane (2). (iii) Membrane expression of various receptors decreases (3). The consequence of phospholipid redistribution is the recognition of apoptotic neutrophils as targets for phagocytosis via a macrophage PS receptor (4, 5). The consequence of decreased expression of functional receptors such as adhesion molecules, phagocytic receptors, formyl-methionyl-leucyl-phenylalanine receptors, or TNF receptors is probably the turning off of neutrophil cellular functions (2, 3, 6, 7). The consequence of membrane blebbing is not known. It has been shown in some cases to result in a decreased expression of membrane receptors via the release of bleb-derived vesicles bearing these receptors (8).

The initial purpose of this work was to investigate the mechanism of apoptosis-dependent CD43 down-regulation during neutrophil apoptosis. Leukosialin (CD43) is the main sialoglycoprotein of the leukocyte plasma membrane, described both as an anti-adhesive and an adhesive molecule (9, 10). Its expression decreases during neutrophil activation and adhesion because of a proteolytic cleavage of the molecule (11–15). During neutrophil apoptosis, CD43 expression also decreases but, as shown here and unlike what had been postulated (3, 7), without proteolysis or internalization of the molecule. We thus investigated the release of membrane vesicles during neutrophil apoptosis in relation to the decreased expression of CD43. Here, for the first time, we analyze the mechanism of apoptosis-induced blebbing in neutrophils. We show that CD43 down-regulation parallels but does not result from membrane blebbing, with CD43 being released on microvesicles distinct from membrane blebs.²

MATERIALS AND METHODS

Reagents—PBS, Dulbecco's modified Eagle's medium, and fetal calf serum were from Invitrogen. BSA, purified goat IgGs, rabbit anti-actin antibody, aprotinin, leupeptin, chymostatin, phenylmethylsulfonyl fluoride, 1,10-phenanthroline, gliotoxin, methylthiogliotoxin, staurosporine, cyanide 4-trifluoromethoxyphenylhydrazine, calibrated 0.1- and 3-µm latex beads, and the PKH26 fluorescent cell linker kit were from

The most obvious initial stages of neutrophil apoptosis occur at the plasma membrane as follows. (i) Cytoskeleton move-

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental material in the form of Videos 1–3 on bleb formation.

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¹ The abbreviations used are: PS, phosphatidylserine; BSA, bovine serum albumin; DIC, differential interference contrast; fmk, fluoromethyl ketone; FSC, forward scatter; mAb, monoclonal antibody; MFI, mean fluorescence intensity; MLCK, myosin light chain kinase; ML9, 1-(5-chloronaphthalene-1-sulfonyl)homopiperazine; pAb, polyclonal antibody; PBS, phosphate buffered saline; PE, phycoerythrin; PKC, protein kinase C; PMA, phorbol myristate acetate; PMN, polymorphonuclear neutrophil; SSC, side scatter; TNF, tumor necrosis factor; TRITC, tetramethylrhodamine isothiocyanate; Z, benzyloxycarbonyl.

² Preliminary data from this work have been presented at the British Society Meeting on Apoptosis in Myeloid Cells, November 2003, Edinburgh, UK (see Ref. 61).

Sigma. The Hemacolor blood smears staining kit was from Merck. PE- or allophycocyanin-annexin V, Viaprobe (7AAD), fluorescein thiocyanate-labeled or unlabeled mouse anti-CD43 mAb (clone G10), allophycocyanin-anti-CD41a(GPIIb), PE-anti-glycophorin A, and corresponding isotypic controls were from BD Biosciences. The fluorescein thiocyanate- or PE-labeled mouse mAbs anti-human CD3, CD11a, CD11b, CD16, CD66b, and CD63 and the corresponding isotypic controls were from Immunotech/Coulter (Marseille, France). TRITC-, peroxidase-, or alkaline phosphatase-labeled anti-mouse IgG or anti-rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). Human recombinant TNF- α was from PreproTech (Rocky Hill, NJ). Z-VAD-fmk, chelerythrine chloride, ML9, and ML7 hydrochloride were from Calbiochem, bisindolylmaleimide I (Gö6850) was from Alexis (San Diego, CA), Z-DEVD-fmk came from Bachem (Bubendorf, Germany), and JC-1 was purchased from Molecular Probes (Eugene, OR). We obtained a polyclonal antibody to the total intracytoplasmic portion of CD43 (anti-CD43cyto pAb) by immunizing a rabbit with the recombinant intracellular portion of CD43 expressed in *Escherichia coli* from a plasmid (16), which was kindly donated by M. Fukuda (La Jolla, CA). The antibody was immunopurified on the recombinant protein.

Neutrophils and Microparticles—EDTA-anti-coagulated blood was centrifuged at room temperature for 20 min at $150 \times g$, and the platelet-rich plasma was further centrifuged for 10 min at $1,000 \times g$ to pellet platelets. Blood cells were then resuspended in the platelet-poor plasma and centrifuged for 35 min at $700 \times g$ on PolymorphPrep (Axis-Shield, Oslo, Norway) to obtain neutrophils. Residual erythrocytes were lysed by a 1-min step in 0.2% NaCl, and neutrophils were distributed in a 24-well PRIMARIA plate (BD Biosciences) at a concentration of 2×10^6 cells/ml in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Cells were maintained at 15°C overnight and then brought to 37°C in a 5% CO_2 incubator to promote apoptosis. Alternatively, fresh cells were incubated at 37°C with gliotoxin and TNF- α , as described (17).

To isolate microparticles as analyzed in Fig. 8, after the usual centrifugation for 10 min at $350 \times g$, which pulled down cells and all the cell-derived vesicles analyzed in Fig. 6, the supernatant was centrifuged for 20 min at $2,000 \times g$. Further ultracentrifugation of the supernatant, for 1 h at $100,000 \times g$ (rotor S55S-609 in a Sorvall RCM150 GX centrifuge), allowed recovery of microparticles.

Flow Cytometry Analysis and Cell Sorting—All media and buffers were previously filtered ($0.2 \mu\text{m}$) in flow cytometry experiments. Neutrophils and the membrane vesicles analyzed in Fig. 6 were spun down at $350 \times g$ and suspended in $10 \mu\text{l}$ of PBS containing 1% BSA and 0.1% sodium azide (PBS-BSA-azide). They were treated at 4°C for 20 min with 1 mg/ml heat-aggregated (30 min at 56°C) goat IgG to block Fc γ receptors and then for 30 min with fluorescein thiocyanate- or PE-labeled anti-CDx antibodies. Washed cells were suspended in the annexin-binding buffer containing 2.5 mM CaCl_2 and treated with PE- or allophycocyanin-labeled annexin V and 7AAD (Viaprobe) according to the manufacturer's instructions. Allophycocyanin-annexin V was required in samples containing bisindolylmaleimide or chelerythrine, which give an FL1 and FL2 background fluorescent signal. Cells were immediately analyzed by flow cytometry on a BD Biosciences FACScalibur. When mentioned, cells were fixed and permeabilized before labeling with the fluorescence-activated cell sorter permeabilizing solution from BD Biosciences according to the manufacturer's instructions.

Membrane vesicles were identified on the basis of forward scatter and side scatter analysis. FSC and SSC channels were set at logarithmic gain to analyze particles of various sizes, and an acquisition threshold was set on the forward scatter to reduce background signals. When mentioned, R2/R3 vesicles (see Fig. 6) were collected using a BD Biosciences FACSvantage cell sorter. All of these sorted vesicles were recovered by a 15-min centrifugation at $1,500 \times g$. They were cytocentrifuged (700 rpm, high acceleration, Shandon cytospin) on poly-L-lysine-coated slides and analyzed by Hemacolor staining.

Microparticles, collected in the $100,000 \times g$ pellet, were labeled as described above with heat-aggregated IgGs and antibodies, which were previously ultracentrifuged for 20 min at $11,600 \times g$ in a microfuge, and analyzed by flow cytometry with an FSC/SSC setting allowing the analysis of $<1 \mu\text{m}$ particles with minimum background signals.

Cell Analysis by DIC and Fluorescence Microscopy—To analyze cell morphology, neutrophils were fixed for 30 min at 4°C in 1% glutaraldehyde and analyzed by differential interference contrast (DIC) light microscopy. The percentage of blebbing cells was determined microscopically by counting at least 100 cells for each condition. Membrane labeling with the membrane fluorescent probe PKH26 was performed according to the manufacturer's instructions for 10 min at 15°C on

neutrophils pre-incubated overnight at this temperature. Cells were washed and then incubated at 37°C to induce apoptosis. Alternatively, neutrophils were labeled with an anti-CD43 or anti-CD11b antibody in PBS-BSA-azide on ice after the 15 and 37°C incubations. They were then fixed for 30 min with 0.05% glutaraldehyde and 3% paraformaldehyde, incubated overnight in blocking solution (PBS-BSA-azide containing 2.5% AB group human serum), and treated with TRITC-labeled goat anti-mouse IgG. They were analyzed by fluorescence microscopy on a Leica DMIRB microscope ($\times 63$ objective) equipped with an Olympus DP11 digital camera.

Cytoentrifuged neutrophils, treated with Hemacolor staining, were observed by light microscopy. The percentage of apoptotic cells with a condensed nucleus was determined microscopically by counting at least 100 cells for each condition.

Electron Microscopy—For morphologic examination, neutrophil samples were fixed with 1.5% glutaraldehyde, washed, post-fixed in OsO_4 , and embedded in Epon. For CD43 and CD11b distribution analysis, neutrophils were labeled at 4°C with anti-CD43 or anti-CD11b mAbs as described above and then labeled with an immunogold-conjugated (10 nm) goat anti-mouse IgG (British Biocell, Cardiff, UK). Cells were then fixed with 1.25% glutaraldehyde for 30 min at 4°C , washed, fixed with osmic acid, alcohol-dehydrated, and finally embedded in Epoxy resin. Thin sections were examined with a Philips CM 10 electron microscope (Philips, Eindhoven, The Netherlands) after uranyl acetate and lead citrate staining.

Time Lapse Fluorescence Microscopy—Membrane labeling with the fluorescent probe PKH26 was performed as described above. Cells were washed at 15°C , brought to 37°C for 30 min, deposited in a microwell dish with glass bottom (Mat Tek Corp. Ashland, MA) coated with poly-L-lysine, and maintained at 37°C . Images were collected every 7 s on a Zeiss fluorescence microscope (Axiovert 135) equipped with a cooled charge-coupled device camera (MicroMax 5 MHz, Princetown Instruments) driven by Metamorph Imaging System software (Universal Imaging). Quick Time movies were accelerated 100-fold.

As a negative control, freshly isolated neutrophils, incubated in fetal calf serum-containing Dulbecco's modified Eagle's medium for 1 h at 15°C and then brought to 37°C for up to 2 h, demonstrated no blebbing transformation when fixed with glutaraldehyde and analyzed by DIC microscopy or when applied on a poly-L-lysine-coated plate and observed directly on the inverted microscope (data not shown).

Western Blotting—The neutrophil pellet was lysed in PBS containing 1% Nonidet P-40, 10 mM EDTA, and the protease inhibitors aprotinin (0.1 trypsin inhibitor unit/ml), leupeptin (100 $\mu\text{g}/\text{ml}$), chymostatin (20 $\mu\text{g}/\text{ml}$), phenylmethylsulfonyl fluoride (1 mM), and 1,10-phenanthroline (1 mM). After a rapid centrifugation at $11,000 \times g$ to remove nuclei, each lysate was supplemented with half its volume of $2\times$ Laemmli SDS-reducing sample buffer and submitted to PAGE and transfer. Microparticle pellets were made soluble directly in boiling sample buffer. Western blots were incubated with an anti-CD43 mAb or the anti-CD43cyto pAb and then with peroxidase-labeled anti-mouse or anti-rabbit IgG and revealed by chemiluminescence. When mentioned, actin was measured on the lower part of the membrane using a rabbit anti-actin antibody and alkaline phosphatase-labeled secondary antibody as revealed by colorimetry. This less sensitive detection assay allowed us to avoid saturation and to obtain concentration-related signals.

Measure of Mitochondria Membrane Potential—This was measured by flow cytometry using the mitochondria fluorescent probe JC-1, as described (18). Briefly, neutrophils were pre-incubated overnight at 15°C , washed in Dulbecco's modified Eagle's medium without serum, incubated at 15°C for 20 min with the mitochondria probe JC-1 (7.7 μM), washed, and then incubated at 37°C to promote apoptosis. They were analyzed at different times by flow cytometry, and the proportion of apoptotic neutrophils with low mitochondria potential is expressed as a percentage of neutrophils with high FL1 mean fluorescent intensity.

Statistical Analysis—Data obtained from apoptotic neutrophils *versus* control cells maintained at 15°C or neutrophils with signaling inhibitors *versus* control neutrophils in medium were compared using a paired *t* test. Statistical significance was defined as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ (Figs. 2, 3, 6–8).

RESULTS

Modulation of Membrane Receptors and Phosphatidylserine Exposure on Apoptotic Neutrophils—As reported previously (7), Fig. 1A shows that CD43 membrane expression decreases during neutrophil incubation for 20 h at 37°C as compared with cells maintained at 4°C . This decrease is not simultaneous in all neutrophils but involves only a subpopulation of CD43^{low}

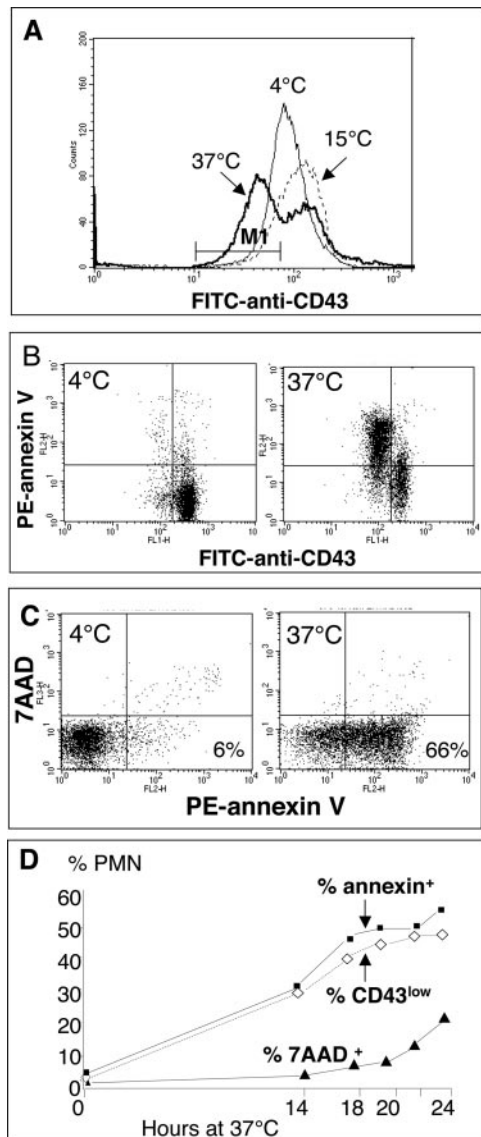


FIG. 1. Flow cytometry analysis of CD43 expression and PS externalization on apoptotic neutrophils. A–C, neutrophils were allowed to apoptose spontaneously overnight at 37 °C or were maintained at 4 °C and then labeled with fluorescein thiocyanate-anti-CD43 (A and B), PE-annexin V (B and C), and 7AAD (Viaprobe) (C). Results are expressed as the percentage of CD43^{low} (M1 in panel A) or annexin⁺ PMN (lower right quadrant in panel C). D, kinetic analysis of the appearance of CD43^{low} (as in panel A), annexin⁺ and necrotic 7AAD⁺ (as in panel C) neutrophils during their spontaneous apoptosis at 37 °C.

neutrophils (M1 = 60% CD43^{low} neutrophils in the example shown in Fig. 1A). This subpopulation of CD43^{low} neutrophils also binds annexin V (Fig. 1B) and thus represents neutrophils undergoing apoptosis. The absence of labeling with the viability probe 7AAD (Fig. 1C) shows that these annexin-binding neutrophils (66% annexin⁺ neutrophils in the example shown here) have intact plasma membranes and are not necrotic cells. As expected, the kinetic appearance of the CD43^{low} population during spontaneous apoptosis at 37 °C paralleled that of the annexin V binding (Fig. 1D).

To analyze the kinetic of these membrane changes, it was convenient to accelerate the neutrophils' spontaneous apoptosis. We used a previously described protocol in which apoptosis is synchronized by maintaining neutrophils overnight at 15 °C before warming them at 37 °C (19). Apoptosis is arrested at 15 °C because of the failure of the insertion of proapoptotic Bax into mitochondria. Reheating at 37 °C results in a burst of

synchronous apoptosis assessed by annexin V binding, cell morphology, a terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling assay, and DNA laddering (19). Neutrophils incubated overnight at 15 °C express the same level of CD43 as freshly isolated cells (data not shown), whereas the 4 °C incubation tends to decrease this expression slightly (Fig. 1A). After overnight incubation at 15 °C and warming at 37 °C, we observed the appearance of CD43^{low} neutrophils that started, together with annexin V binding, after 45–60 min of incubation at 37 °C and reached a plateau of $50 \pm 20\%$ apoptotic neutrophils after 1.5–2 h (Fig. 2A). CD43 down-regulation and PS externalization appeared as simultaneous events, whereas mitochondria membrane depolarization, assessed by the fluorescent shift of mitochondria probe JC-1 (18), started earlier, after 20–30 min of incubation (Fig. 2B).

Another protocol used to accelerate neutrophil apoptosis was the induction of apoptosis by TNF- α in the presence of the NF- κ B inhibitor gliotoxin (17). When freshly isolated neutrophils were incubated at 37 °C with 10 ng/ml TNF- α and 0.1 μ g/ml gliotoxin, a population of annexin-binding and CD43^{low} neutrophils appeared after 1.5 h of incubation (data not shown). These cell changes did not occur when gliotoxin was replaced by its inactive analogue methylthiogliotoxin.

Fig. 2C shows the modulation of different markers on neutrophils pre-incubated overnight at 15 °C and then for 2 h at 37 °C as compared with control neutrophils maintained at 15 °C. As mentioned above, the expression of CD43 was decreased as was CD16 (receptor III for the Fc portion of IgG, also known as Fc γ RIII) expression, which is known to be down-regulated on apoptotic neutrophils (2, 20), whereas we observed a significant increase of CD11b and a stable expression of CD11a.

CD43 Decreased Expression Is Not Due to a Proteolytic Cleavage or Endocytosis—Cell lysates were analyzed by Western blot using either an anti-CD43 mAb that recognizes an extracellular epitope or a polyclonal antibody raised against the whole cytoplasmic domain of CD43 (anti-CD43cyto pAb) (Fig. 3A). This anti-CD43cyto pAb is a useful tool that makes it possible to visualize all cell-associated CD43 fragments even if the extracellular part of the molecule is cleaved or desialylated (3). In freshly isolated neutrophils lysed in the presence of protease inhibitors (Fig. 3B) or immediately boiled in reduced sample buffer (data not shown), the anti-CD43cyto pAb recognized the whole 140-kDa CD43 molecule designed by the anti-CD43 mAb as well as three minor 110–95-kDa bands, that could represent incompletely glycosylated CD43 or limited fragmentation during cell solubilization. Neutrophils incubated overnight at 15 °C showed the same Western blot CD43 pattern as freshly isolated cells, and no further cleavage occurred during the 2-hour apoptosis stage at 37 °C. By contrast, neutrophil activation by PMA resulted in cell-associated CD43 fragments (a major 95-kDa doublet and a 26-kDa band), confirming previous reports (14, 15). The intensities of CD43 scanned bands, normalized for the actin content of the same samples, showed a significant decrease of the native CD43 band during synchronized apoptosis of neutrophils at 37 °C (Fig. 3C). The same decrease was observed in blots revealed by the anti-CD43cyto pAb recognizing the whole intracellular domain ($p = 0.02$, $n = 4$) and by the anti-CD43 mAb recognizing the extra-cellular portion of the molecule ($p < 0.005$, $n = 5$). Therefore, CD43 seems to be shed as an entire transmembrane molecule together with surrounding membrane structures. It is worth noting that the band immediately below the CD43 band, recognized by the anti-CD43cyto pAb but not by the anti-CD43 mAb, also decreases during apoptosis (data not shown).

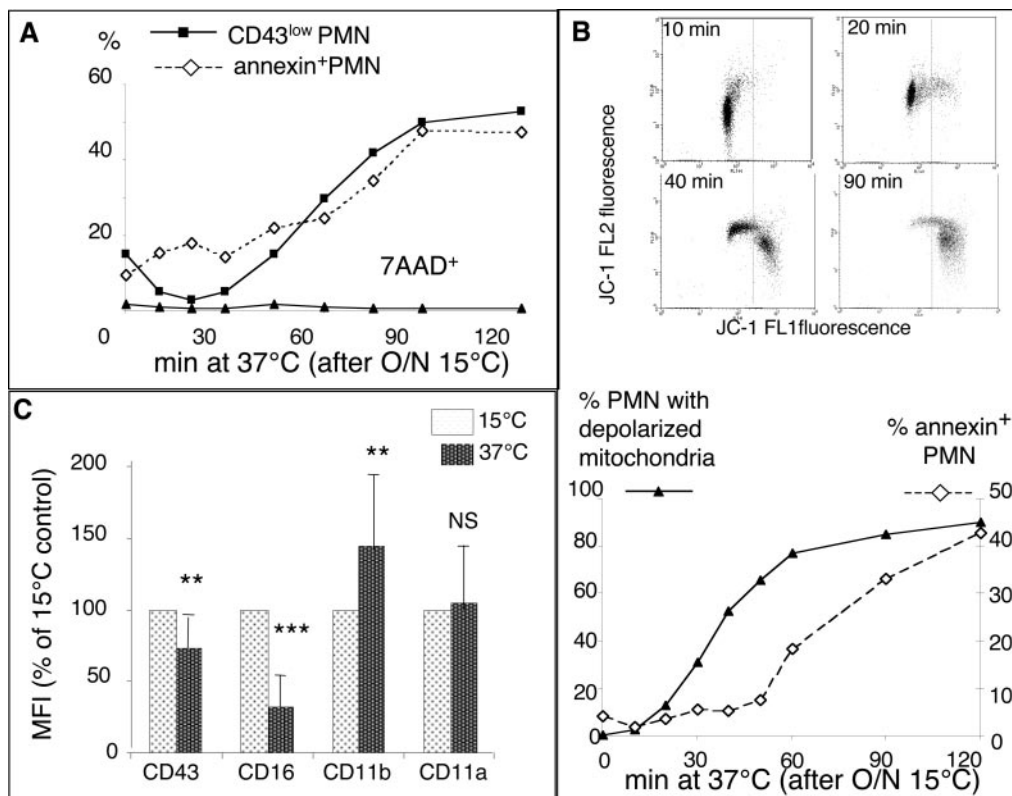


FIG. 2. Flow cytometry analysis of membrane markers, annexin binding, and mitochondria depolarization during synchronized apoptosis of neutrophils. Neutrophils were pre-incubated overnight at 15 °C and then brought to 37 °C in 5% CO₂. A, kinetic analysis as in Fig. 1D. B, after overnight (O/N) incubation, neutrophils were treated at 15 °C with the mitochondrial probe JC-1 and then incubated at 37 °C to promote apoptosis. PMNs with depolarized mitochondria show an enhanced FL-1 fluorescence. Parallel cell samples were analyzed for annexin V binding as in Fig. 1. C, neutrophil expression of each receptor is given as the MFI obtained for each fluorescently labeled anti-CD mAb with cells incubated for 2 h at 37 °C (MFI at 37 °C × 100/MFI of control cells maintained at 15 °C). Means ± S.D. of 10 to 15 experiments were compared statistically. **, $p < 0.01$; ***, $p < 0.001$; NS, not significant.

The absence of CD43 internalization was further investigated by flow cytometry analysis of fixed and permeabilized or unpermeabilized cells. Fig. 3D shows the results of three experiments in which the decrease of CD43 labeling after a 2-hour apoptosis at 37 °C was exactly the same, regardless of whether anti-CD43 mAbs could penetrate inside the cells or not. As a control for cell permeabilization, anti-myeloperoxidase antibodies strongly labeled permeabilized, but not unpermeabilized, neutrophils (data not shown). We can conclude from these results that the decrease of CD43 membrane expression, initially observed by flow cytometry on apoptotic neutrophils, was not due to the fragmentation, desialylation, or internalization of the CD43 molecule.

Fixed and Time Lapse Microscopy Analysis of the Blebbing Process—The shedding of the entire CD43 molecule implies a release of CD43-bearing membrane vesicles. An early membrane effect of apoptosis that could result in the release of such vesicles is the blebbing of the plasma membrane. We confirmed that only a few cells are in this blebbing stage at any given time during the spontaneous apoptosis of neutrophils at 37 °C (data not shown). However, when neutrophils were synchronized by overnight pre-incubation at 15 °C, we then observed numerous blebbing cells after warming at 37 °C, either by DIC microscopy (Fig. 4A, arrows) or fluorescent microscopy after membrane labeling using the PKH26 fluorescent probe (21) (Fig. 4B). Blebbing cells were first detected after 20 min at 37 °C, and their number increased progressively to reach ~50% of neutrophils after a 1-h incubation (Fig. 4D). After 1.5 h, this number decreased to <15% of blebbing cells after 2 h. Cells, which had finished their blebbing reaction, appeared round and with a smoother surface as compared with cells kept at 15 °C (Fig. 4A,

2.5 h). The diffusion into the cell of the PKH26 fluorescent label made it possible to distinguish nuclear lobes with condensed chromatin in perfectly round cells (Fig. 4B, 2 h, arrowheads). This observation was confirmed by Hemacolor staining (Fig. 4C), showing that the number of neutrophils with a condensed nucleus was low before 30 min of incubation and increased regularly to reach 80% of neutrophils after 2 h (Fig. 4D).

Similar results were obtained with TNF- α and gliotoxin-promoted apoptosis; >50% blebbing neutrophils appeared after a 1.5–2-h incubation together with the annexin V binding (data not shown). No blebbing was observed when gliotoxin was replaced by methylgliotoxin.

Membrane labeling with PKH26 of cells pre-incubated at 15 °C and then brought to 37 °C made it possible to follow bleb formation by time lapse fluorescent microscopy (Fig. 4E: Videos 1, 2 and 3 are available as supplemental material in the on-line version of this article). Video 1 shows the dynamic of reversible bleb formation and re-absorption on two cells undergoing apoptosis as an initially resting round cell begins its blebbing stage. Video 2 shows a morphological transformation sequence of an apoptotic neutrophil with the clear detachment of a bleb-derived membrane vesicle. Video 3 shows that these membrane vesicles remain attached to the cell body by long membrane filopodia.

CD43 and CD11b Distribution Determined by Fluorescent and Electron Microscopy Analysis of Membrane Blebs—An electron microscopy analysis of neutrophils incubated overnight at 15 °C and then for 1 h at 37 °C clearly showed neutrophils with a normal nucleus but an irregular blebbing surface (Fig. 5A). At higher magnification, membrane blebs appeared deprived of granules and organelles (Fig. 5, B and D). Narrow cavities, which are sometimes observed in a row along the “root” of the

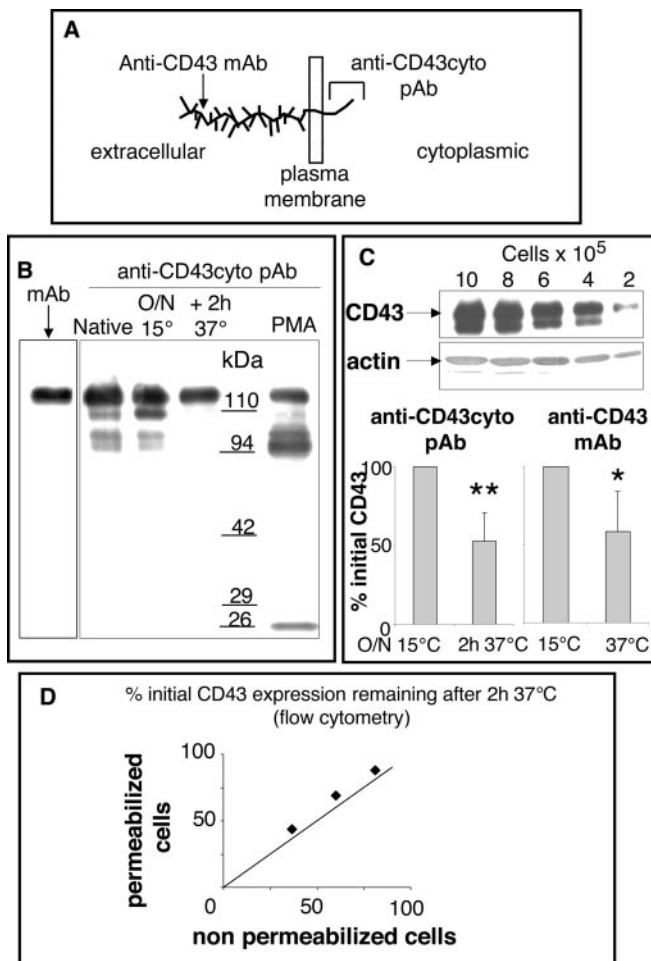


FIG. 3. Qualitative and quantitative analysis of CD43 on apoptotic neutrophils: *A*, schematic representation of the transmembrane CD43 molecule showing anti-CD43 mAb and anti-CD43cyto pAb binding sites. *B*, cell lysates from freshly isolated neutrophils (*Native*), neutrophils kept at 15 °C overnight (*O/N 15°*) and then for 2 h at 37 °C (+ 2h 37°) or activated for 30 min at 37 °C with 10 ng/ml PMA (*PMA*), were separated by SDS-PAGE on 5–15% acrylamide gradient and analyzed by Western blot using either the anti-CD43 mAb (native cells) or the anti-CD43cyto pAb. *C*, serial dilutions of cell lysates were analyzed by Western blot with anti-CD43cyto pAb and anti-actin antibodies. Intensities of scanned CD43 and actin bands from cells pre-incubated overnight (*O/N*) at 15 °C with or without a further 2-hour incubation at 37 °C were plotted and compared. Results are expressed as the percentage of the CD43 band intensity measured in control cells at 15 °C and normalized with actin (mean $\pm$ S.D. of 4 or 5 experiments, see “Results”). *, $p < 0.05$; **, $p < 0.01$. *D*, CD43 expression was measured by flow cytometry on neutrophils permeabilized or not as described under “Materials and Methods.” Results are expressed as the MFI of cells incubated for 2 h at 37 °C $\times$ 100/MFI of control cells at 15 °C.

blebs (Fig. 5*B*, arrows) and suggest that the blebs are about to detach from the cell body, had been already observed *in vivo* (22).

Fluorescent microscopy revealed that membrane blebs observed by DIC microscopy (Fig. 5*C*, sections *a'* and *b'*, arrowheads) were not labeled with anti-CD43 mAbs (Fig. 5*C*, sections *a* and *b*), whereas anti-CD11b mAbs underlined the cell contour, including membrane blebs (Fig. 5*C*, sections *c* and *c'*).

Pre-embedding immunogold labeling for CD43 (Fig. 5, *D* and *E*) confirmed this observation and showed that, whereas CD43 was evenly distributed all along the cell surface (Fig. 5*D*, insets *D1* and *D4*), blebs were mostly devoid of gold particles (Fig. 5*D*, insets *D2* and *D3*, and Fig. 4*E*). By contrast, gold particles bearing anti-CD11b mAbs underlined the entire cell surface, including blebs (Fig. 5*F*).

Flow Cytometry Characterization of Membrane Vesicles Released during the Blebbing Stage of Neutrophil Apoptosis—Because some of these blebs detach from the cell body (supplemental material Videos 2 and 3), we then attempted to individualize, by flow cytometry, vesicles released by apoptotic neutrophils. Side scatter and forward scatter analysis of neutrophil suspensions kept at 15 °C and then incubated for 2 h at 37 °C made it possible to distinguish seven distinct vesicle populations (Fig. 6*A*). After multiple labeling, vesicle populations were tested for the expression of markers defining their leukocyte origin (Table I). The R1 region contained neutrophils. The R6 region contained contaminating lymphocytes (CD3 positive), whereas R7 mostly contained erythrocyte-derived (glycophorin A positive) and, when present, platelet-derived vesicles (GPIIb positive). The R2 to R5 regions contained particles bearing neutrophil markers CD11b and CD66b. The number of particles in R4, representing mostly <100 nm vesicles, only slightly increased during neutrophil incubation at 37 °C. The R5 region contained large vesicles whose number did not change significantly during neutrophil temperature-induced apoptosis (Fig. 6*B*). Finally, two distinct vesicle populations in the R2 and R3 regions appeared clearly during incubation at 37 °C (Fig. 6*B*), and their sizes, ranging from one-fourth to one-twentieth the size of a neutrophil, were similar to those of the blebs observed by microscopy. They were identified as plasma membrane vesicles, because they retained the membrane marker PKH26 (data not shown). These vesicles bound annexin V and bear the neutrophil markers CD11b and CD66b (Table I). To quantitate the level of CD43 expression of each vesicle population, we expressed the anti-CD43 mean fluorescence intensity against the size (mean FSC). The results of normalized values for neutrophil density (density index, as defined in Table I legend) confirmed that bleb-derived R2 vesicles were poorly labeled with anti-CD43 as shown by electronic microscopy data (Fig. 5), whereas CD43 positive vesicles from the R4 population were enriched in CD43, as discussed below.

One should point out that, despite their different sizes, all of the vesicles shown in Fig. 6 became sedimented with neutrophils after a standard centrifugation at $350 \times g$. Attempts to isolate released blebs from neutrophils by step centrifugations or centrifugation on Percoll gradients were unsuccessful. Cytometry cell sorting, based on side scatter/forward scatter dot plots, finally isolated vesicles appearing at 37 °C in the R2 and R3 regions. After cyto-centrifugation they appeared as similarly round vesicles by Hemacolor staining, taking the pink marker for cytoplasm but lacking nucleus or nucleus fragments (Fig. 6*C*). The release of R2 and R3 vesicles followed similar kinetics. Fig. 6*D* shows, for convenience, the sum of R2 and R3 released vesicles (designated R2/R3), which paralleled that of the CD43 expression decrease or the appearance of annexin-binding neutrophils; the number of R2/R3 vesicles increased after 45 min at 37 °C with a plateau at 90–120 min.

Apoptosis Membrane Events Involve Distinct Signaling Pathways—Various signaling inhibitors were added to neutrophils to assess whether PS “flip-flop”, CD43 decreased expression, membrane blebbing, and R2/R3 vesicle release result from similar mechanisms. Similar results were obtained whether inhibitors were constantly present during the 15 °C pre-incubation followed by a 2-h incubation at 37 °C or when added only during the secondary incubation at 37 °C.

Phosphatidylserine Externalization—The specific caspase-3 inhibitor Z-DEVD-fmk (Fig. 7*A*) and the pan-caspase inhibitor Z-VAD-fmk (data not shown) significantly prevented the phospholipid scrambling that occurred after neutrophil incubation at 37 °C and was revealed by annexin V binding ($n = 7$ experiments). This was also the case for PKC inhibitors bisindolyl-

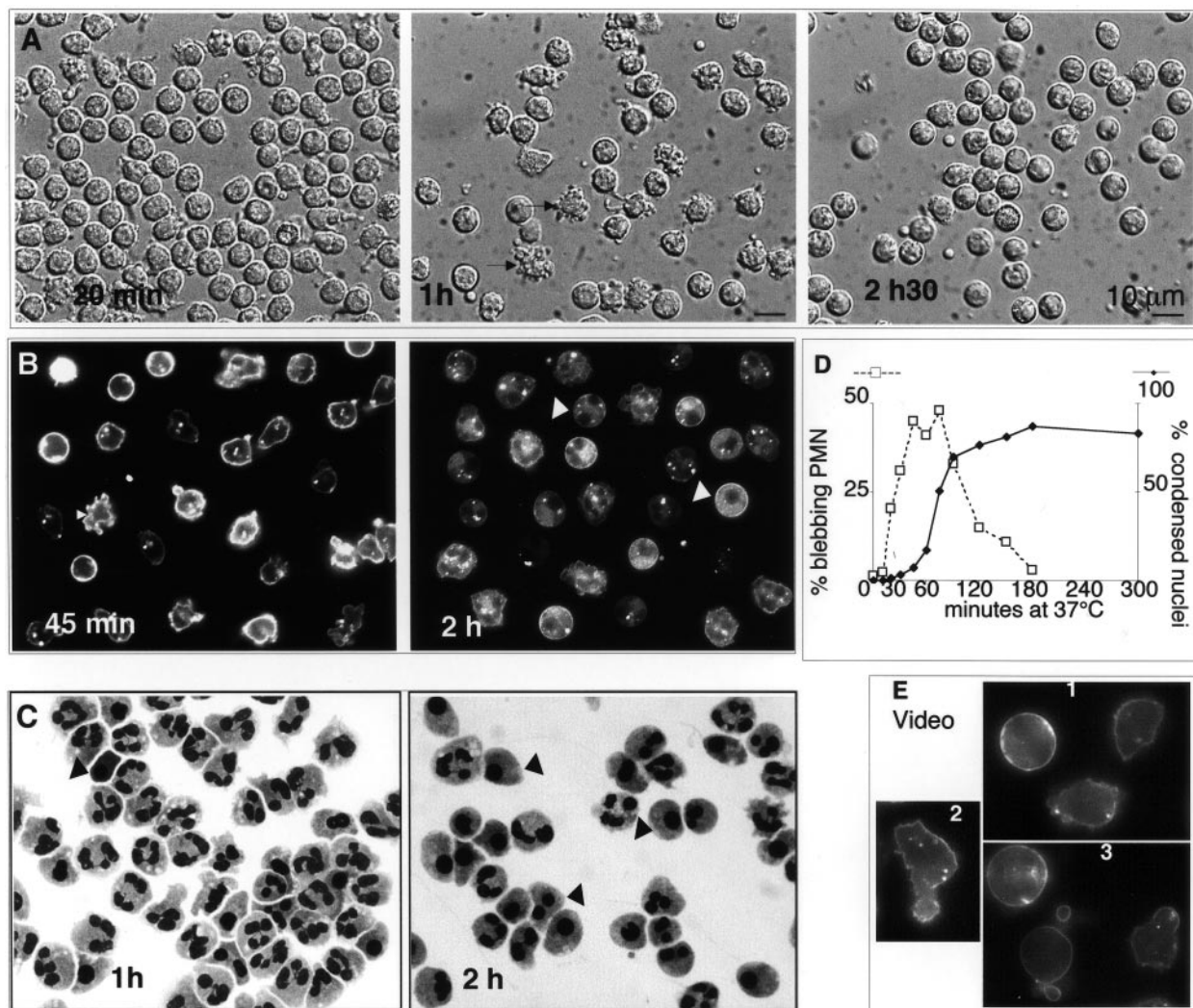


FIG. 4. Microscopic analysis of the membrane blebbing stage. Neutrophils pre-incubated overnight at 15 °C were incubated at 37 °C, fixed with glutaraldehyde at different times, and analyzed by DIC (A), labeled with membrane fluorescent probe PKH26 and then brought to 37 °C and analyzed at different time by fluorescence microscopy (B and E), or incubated as in panel A and then cytocentrifuged, fixed in methanol, and stained with Hemacolor staining (C). Arrows show blebbing neutrophils, whereas arrowheads show apoptotic neutrophils with condensed nucleus. Fig. 3D shows a kinetic profile of experiments analyzed in panels A and C expressed as the percentage of blebbing PMN or the percentage of PMN with condensed nucleus. Videos of panel E are available as supplemental material in the on-line version of this paper. Scale bar, 10 μ m.

maleimide I (Gö6850) (Fig. 7A, $n = 8$ experiments), chelerythrine chloride ($78 \pm 7\%$ and $64 \pm 8\%$ annexin⁺ neutrophils after a 2-hour incubation at 37 °C without and with chelerythrine, respectively; $p = 0.001$, 7 experiments) and staurosporine (data not shown). By contrast, the MLCK inhibitors ML9 (Fig. 7A) and ML7 (data not shown) had no significant effect on the annexin V binding.

Blebbing and Release of Bleb-derived Vesicles—The caspase-3 inhibitor Z-DEVD-fmk (Fig. 7B, $n = 8$ experiments) did not prevent the release of apoptosis-related vesicles R2/R3, which was even significantly increased by the pan-caspase inhibitor Z-VAD-fmk (data not shown). Similarly, microscopy analysis revealed that neither Z-DEVD-fmk (Fig. 7C) nor Z-VAD-fmk (data not shown) inhibited cell membrane blebbing. Z-DEVD-fmk and Z-VAD-fmk even increased the number of blebbing neutrophils incubated overnight at 37 °C without the 15 °C synchronizing step (data not shown), presumably because they do not allow apoptosis to proceed and stop the cells at the blebbing stage (23, 24). PKC inhibition by bisindolylmaleimide (Fig. 7B, $n = 6$ experiments), chelerythrine, or staurosporine (data not shown) significantly increased the number of released R2/R3 vesicles and did not prevent the blebbing (Fig.

7C). By contrast, ML9 strikingly inhibited the release of R2/R3 vesicles (Fig. 7B) and prevented cell blebbing, as shown by microscopy analysis (Fig. 7C). Similar results were obtained with ML7 ($n = 4$ experiments, data not shown).

Decrease of CD43 Membrane Expression—The caspase-3 inhibitor Z-DEVD-fmk inhibited CD43 down-regulation (Fig. 7D, anti-CD43 MFI was 186 ± 14.4 with Z-DEVD-fmk and 130 ± 25 for control cells incubated at 37 °C for 2 h, $p < 0.001$, $n = 11$ experiments). By contrast, the pan-caspase inhibitor Z-VAD-fmk further decreased CD43 expression (anti-CD43 MFI was 79.9 ± 27.7 with Z-VAD and 110.0 ± 24.5 for control cells incubated at 37 °C for 2 h; $p = 0.02$, $n = 10$ experiments). Neither bisindolylmaleimide (Fig. 7D) nor chelerythrine or staurosporine (data not shown) had any effect on CD43 down-regulation. Finally, MLCK inhibition by ML9 (Fig. 7D) or ML7 (data not shown) did not prevent the decrease of CD43 expression ($65.6 \pm 21.3\%$ of CD43^{low} PMN with ML9 as compared with $61.2 \pm 23\%$ for control cells after 2 h at 37 °C, $n = 21$ experiments).

Released Microparticles Could Account for CD43 Down-regulation—When analyzing the CD43 density on vesicles co-sedimenting with neutrophils (Table I), we noticed that CD43 was

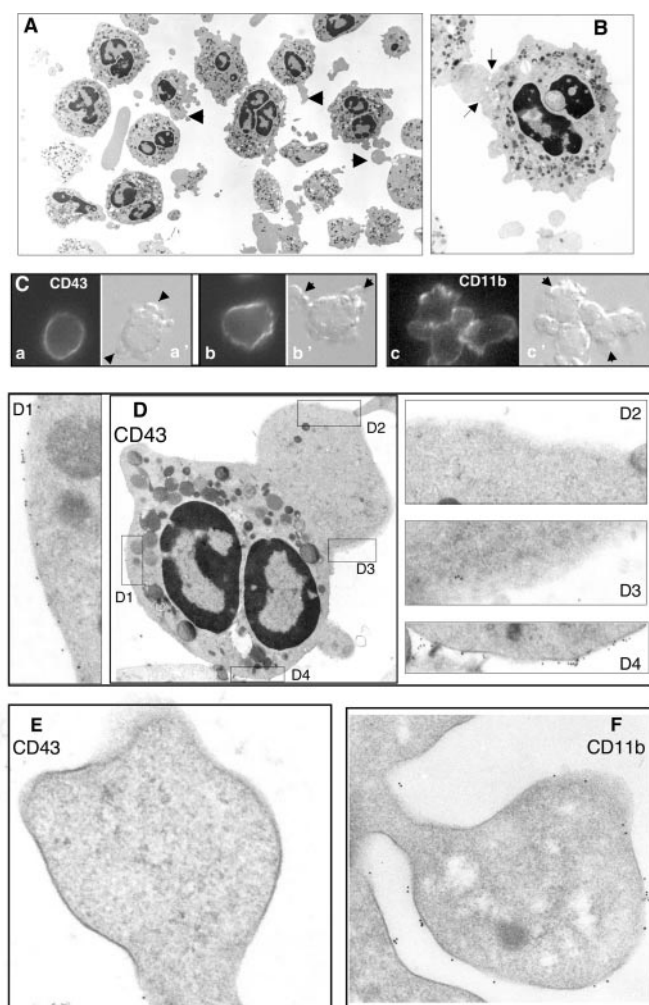


FIG. 5. Electron and fluorescent microscopy analysis of CD43 and CD11b distribution on blebbing neutrophils. A and B, neutrophils pre-incubated overnight at 15 °C and then for 1 h at 37 °C were treated for electron microscopy as described under "Materials and Methods" for morphologic examination at $\times 1460$ (A) and $\times 3700$ (B). C, blebbing neutrophils were labeled with anti-CD43 or anti-CD11b mAbs and TRITC-anti-mouse IgGs, fixed with glutaraldehyde/paraformaldehyde as described under "Materials and Methods," and analyzed by fluorescent microscopy (sections a, b, and c) or DIC light microscopy (sections a', b', and c'). Arrowheads point to labeled or unlabeled blebs; scale bar, 10 μ m. D–F, blebbing neutrophils were labeled with anti-CD43 (D and E) or anti-CD11b (F) mAbs and immunogold-conjugated anti-mouse IgGs. Cells were treated for electron microscopy as described under "Materials and Methods" for CD43 and CD11b distribution analysis at $\times 9,700$ (D), $\times 25,900$ (insets D1–4), and $\times 20,000$ (E and F). All panel D insets and images in panels E and F were further enhanced twice with Adobe Photoshop software to distinguish gold particles.

significantly enriched on the smallest R4 vesicle population when compared with native neutrophils. However, these CD43-rich vesicles represented only $14 \pm 4\%$ of the R4 population (Table I) and could not by themselves account for the decrease of neutrophil membrane expression. This observation, as well as various reports on microparticles released by neutrophils during cell activation, prompted us to search for CD43-rich microparticles in the cell supernatant. Further centrifugation of the cell supernatant at $100,000 \times g$ resulted in a pellet that contained $\leq 1 \mu$ m microparticles as shown by FSC/SSC dot blots of the flow cytometry analysis (Fig. 8A). Double labeling experiments revealed that all CD43 positive particles also bear CD11b, CD63 (Fig. 8A), CD66b and annexin-binding phosphatidylserine (data not shown). They appeared during neutrophil incubation at 37 °C, after overnight 15 °C pre-incubation

(Fig. 8A) and were minimally labeled with isotopic control IgGs (data not shown). CD43 was enriched about 10 times on these particles, when compared with native neutrophils: Indeed CD43 mean fluorescence intensity of labeled particles was 74% of the native neutrophil CD43 MFI, whereas these particles measured as a mean 7% of the neutrophil size as determined by forward scattered analysis (mean of 4 experiments, data not shown).

Because the decrease of neutrophil CD43 expression during apoptosis was inhibited by the caspase-3 inhibitor Z-DEVD-fmk (Fig. 7D), we tested the effect of this inhibitor on the amount of released CD43 positive microparticles measured with calibrated 3- μ m dextran beads as described (25). This release was indeed significantly prevented by Z-DEVD-fmk (Fig. 8B; $n = 4$ experiments, $p = 0.016$). By contrast, it was not modified by the MLCK inhibitor ML9 (5 experiments, $p = 0.42$).

As shown in Fig. 8C, little CD43 was observed in the pellet resulting from a $2,000 \times g$ centrifugation of the cell supernatant that would pull down all remaining cells and R2/R3 bleb-derived vesicles (see cell sorting method). The largest amount of CD43 was recovered in the pellet of the further ultracentrifugation at $100,000 \times g$. Some of the CD43 molecules in this $100,000 \times g$ pellet were cleaved, resulting in a 26-kDa CD43 fragment similar to that observed on PMA-activated cells (Fig. 3B) (14).

DISCUSSION

Membrane blebbing is barely detectable during neutrophil constitutive apoptosis due to the asynchronous apoptosis of cells and the short blebbing stage. This difficulty was efficiently circumvented by accelerating the apoptosis time course by pre-incubating neutrophils at 15 °C (19) or by inducing apoptosis with TNF- α while blocking NF κ B with gliotoxin (17). In these situations, up to 50% of blebbing neutrophils were observed during a short, 1-h period during which PS externalization and CD43 down-regulation were also observed. Repeated kinetic analysis did not allow us to determine whether one of the three membrane events, namely blebbing, phospholipid scrambling, or CD43 down-regulation, occurred first and was likely to trigger the others. All three events occurred simultaneously 15–30 min after mitochondria depolarization and clearly before nuclear chromatin condensation.

Flow cytometry FSC/SSC analysis made it possible to individualize two populations of membrane vesicles (R2/R3) that most probably are released blebs for the following reasons. (i) They bear specific neutrophil membrane markers. (ii) Their release parallels the appearance of membrane blebs observed by microscopy. (iii) Their sizes (one-twentieth to one-fourth the size of a neutrophil) are similar to those of blebs observed by microscopy. (iv) Their release is modulated in the same way as the blebbing by signaling inhibitors, *i.e.* not inhibited by caspase inhibitors or PKC inhibitors but prevented by the MLCK inhibitor, as discussed below.

Although neutrophil apoptosis has mainly been studied *in vitro*, membrane blebbing has also been observed *in vivo* by Shi *et al.*, who analyzed apoptotic neutrophils trapped in hepatic sinusoids following experimental bacteriotoxemia in rats (22). Six to twelve hours after intravenous injection of streptococcal bacteria, they described budding neutrophils in the lumen of sinusoids and observed that the buds were expelled in the early stages of apoptosis before the formation of apoptotic bodies. They showed electronic microscopy images of buds without cellular organelles that are very similar to the images shown here. This *in vivo* observation was important to exclude the possibility that the impressive morphological changes that we observed during the blebbing stage of apoptosis were not an *in*

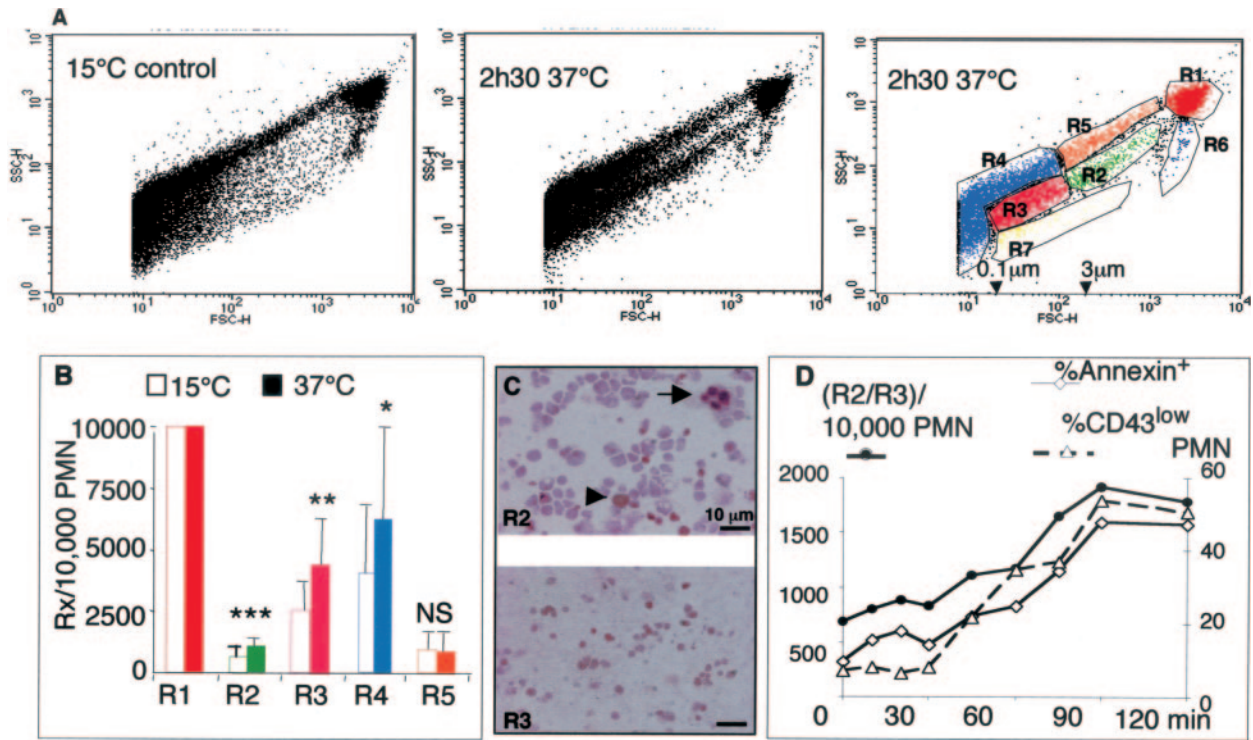


FIG. 6. Flow cytometry analysis of membrane vesicles released during neutrophil apoptosis. A, scatter dot plot of neutrophils maintained at 15 °C overnight and incubated at 37 °C for 2.5 h (2h30). FSC and SSC channels were set at logarithmic gain to analyze particles of various size. 0.1- and 3-µm latex beads were used for calibration (arrowheads). Various FSC/SSC regions were defined, which are underlined with colors. B, in each Rx region the particles were quantified and expressed as the number of particles contained in Rx per 10,000 neutrophils (present in the R1 region), in neutrophil suspension maintained at 15 °C (light color), or incubated for 2 h at 37 °C (dark color). *, $p < 0.05$; **, $p < 0.01$; NS, not significant. C, Hemacolor staining of cytoцентрифугed R2 and R3 vesicles isolated by flow cytometer cell sorting (scale bar, 10 µm) with a contaminating erythrocyte (arrowhead) and a blebbing neutrophil (arrow). D, kinetic analysis of the number of particles appearing in the R2 + R3 regions per 10,000 neutrophils in relation to the percentage of annexin⁺ or CD43^{low} PMN during neutrophil incubation at 37 °C (after incubation overnight at 15 °C).

TABLE I
Expression of membrane markers on membrane vesicles released during neutrophil apoptosis

After pre-incubation overnight at 15 °C then incubation for 2 h at 37 °C, neutrophils and vesicles, spun down at $350 \times g$, were labeled with fluorescent anti-CDx mAbs or with annexin V. Vesicle populations present in the cell suspension, defined in Fig. 6, were analyzed separately. Results are expressed as the percentage of vesicles contained in each FSC/SSC region labeled with a given mAb or with annexin V (mean $\pm$ S.D. of four experiments). Boldfaced numbers show the most common marker of each leukocyte population: CD11b and CD66b for neutrophils, CD3 for T lymphocytes, glycophorin C for erythrocytes, and GPIIb for platelets. CD43 density is expressed as $d_{Rx} = \text{CD43 MFI} / \text{CD43 mean FSC for CD43 positive vesicles from the Rx population}$. The density index, used to compare each population with neutrophils (R1), is $i_{Rx} = (d_{Rx} / d_{R1}) \times 100$.

FSC/SSC regions (neutrophils)	Annexin	CD11b	CD66b	CD3	Glycophorin	GPIIb	CD43	CD43 density index
	%	%	%	%	%	%	%	
R1	56 $\pm$ 21	99 $\pm$ 0.3	100 $\pm$ 0.1	5 $\pm$ 3	3 $\pm$ 5	3 $\pm$ 2	100	100
R2	63 $\pm$ 18	78 $\pm$ 14	63 $\pm$ 13	4 $\pm$ 2	20 $\pm$ 19	2 $\pm$ 1	52 $\pm$ 14	25 $\pm$ 3
R3	46 $\pm$ 17	75 $\pm$ 16	65 $\pm$ 15	6 $\pm$ 5	7 $\pm$ 1	5 $\pm$ 4	35 $\pm$ 7	96 $\pm$ 18
R4	68 $\pm$ 10	61 $\pm$ 21	71 $\pm$ 16	3 $\pm$ 2	3 $\pm$ 0.6	2 $\pm$ 1	14 $\pm$ 4	263 $\pm$ 69
R5	84 $\pm$ 8	75 $\pm$ 15	92 $\pm$ 3	7 $\pm$ 1	3 $\pm$ 2	6 $\pm$ 1	55 $\pm$ 11	122 $\pm$ 27
R6	56 $\pm$ 21	26 $\pm$ 6	23 $\pm$ 7	53 $\pm$ 21	25 $\pm$ 23	4 $\pm$ 5	73 $\pm$ 3	
R7	30 $\pm$ 22	33 $\pm$ 17	34 $\pm$ 15	2 $\pm$ 1	57 $\pm$ 24	5 $\pm$ 6	15 $\pm$ 3	

vitro artifact. Membrane blebbing mainly appears as a reversible process, and time lapse video microscopy shows a re-absorption of most blebs in the cell body. Some blebs, however, result in distinct vesicles that remain loosely attached to the cell by long membrane filopodia. This probably explains our difficulty in separating these vesicles from whole cells by simple centrifugation techniques, despite the size differences. Flow cytometer cell sorting, however, made it possible to isolate these round vesicles that are devoid of nuclei and, therefore, clearly distinct from apoptotic bodies. One could speculate that these vesicles, released at the early stage of apoptosis, could signal the presence of an apoptotic cell, by analogy with the B lymphocyte bleb-derived vesicles reported to be chemotactic for monocytes (8). Bearing external PS, blebs could be engulfed by

macrophages and efficiently trigger anti-inflammatory signals, as do apoptotic cells (26).

Three types of inhibitors, caspase inhibitors, PKC inhibitors, and MLCK inhibitors were used to assess whether phospholipid flip-flop, CD43 down-regulation, blebbing, and R2/R3 vesicle release resulted from common or distinct signaling pathways. An initial screening had shown that these membrane events were not modulated by the tyrosine kinase inhibitor genistein and the P38 mitogen-activated protein kinase inhibitor SB203580, confirming previous data (27) (data not shown). The two schematic signaling pathways for apoptosis (28), the extrinsic pathway of the caspases cascade, and the intrinsic pathway of mitochondria cytochrome C release and apoptosome formation finally result in activation of the same effector

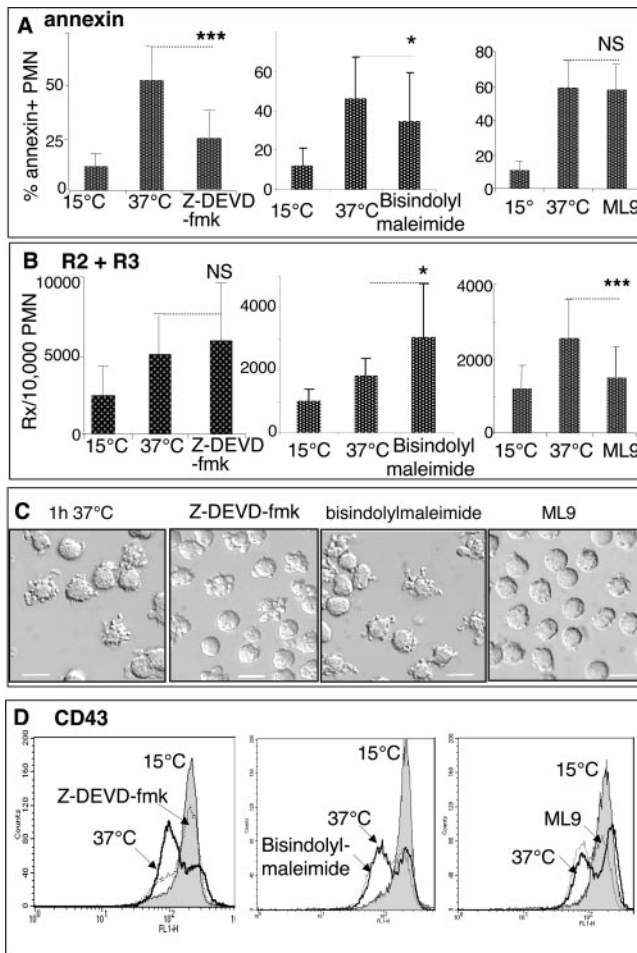


FIG. 7. Effects of caspase PKC and MLCK inhibition on apoptosis-related membrane events. A and B, neutrophils were preincubated at 15 °C overnight and then warmed up for 1.5 h at 37 °C in the presence of 50 μ M Z-DEVD-fmk, 5 μ M bisindolylmaleimide, or 50 μ M ML9. PS externalization (A), membrane vesicles release (B), and CD43 neutrophil expression (D) were measured as described under "Materials and Methods." *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, not significant. C, cells were fixed with glutaraldehyde and analyzed by DIC microscopy for membrane blebbing. Scale bar, 10 μ m. D, representative histograms from six (Z-DEVD-fmk), five (bisindolylmaleimide), and 20 (ML9) similar experiments.

caspases. In neutrophils, which do not contain caspase-6 and caspase-7 (29), caspase-3 is the unique effector caspase, and its activation results in apoptotic DNA/nuclear condensation (30). The two apoptotic pathways are operating in neutrophils. Indeed, neutrophil constitutive apoptosis is thought to be mainly an intrinsic cell process, but it is also modulated by extrinsic signals, *i.e.* accelerated by TNF- α , the Fas ligand, or the TNF-related apoptosis-inducing ligand (31–33) and inhibited by pro-inflammatory mediators such as granulocyte/macrophage colony-stimulating factor or lipopolysaccharide (34, 35). Several reports have demonstrated that caspase-3 causes membrane blebbing via the cleavage of Rho-activated serine/threonine kinase-1, which phosphorylates the myosin light chain (36–38). On the other hand, caspase inhibition has been shown to block the death but not the blebbing of serum-deprived cell lines so that cells "entered into and remained in the execution phase of apoptosis, measured by cell blebbing" (24) without progressing to further stages revealed by DNA laddering and chromatin condensation (23, 24). We herein confirm that the pan-caspase inhibitor Z-VAD-fmk and the specific caspase-3 inhibitor Z-DEVD-fmk do not prevent neutrophil blebbing. Supporting our hypothesis that R2/R3 vesicles are blebs that detached from the

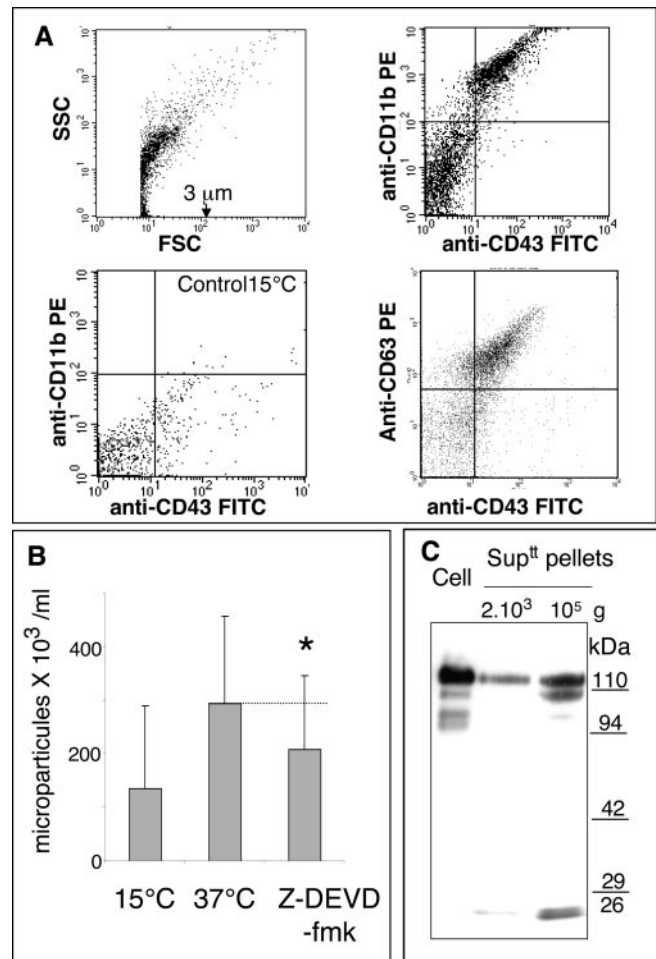


FIG. 8. Analysis of CD43 expression on microparticles released during neutrophil apoptosis. A, flow cytometry analysis of microparticles recovered from the 100,000 $\times g$ pellet of neutrophil supernatant after overnight incubation at 15 °C (control) followed by a 2-hour incubation at 37 °C (all other dot blots). The FSC/SSC dot blot analyzes the size, whereas double labeling analyzes CD43 together with CD11b or CD63 expression. B, the number of CD43 positive microparticles, present in neutrophil supernatant after overnight incubation 15 °C and 2 h at 37 °C, with or without Z-DEVD-fmk, was quantitated with calibrated dextran beads (see "Results"). *, $p < 0.05$. C, Western blot analysis, using the anti-CD43cyto pAb, of whole cell lysate obtained after incubation overnight at 15 °C and for 2 h at 37 °C (Cell) and of pellets obtained after sequential centrifugation of the cell supernatant at 2,000 $\times g$ and 100,000 $\times g$.

cell body, we observed that caspase inhibition did not prevent the release of these vesicles. One should point out that caspase-independent apoptosis involving serine proteases has also been described (39, 40).

CD43 down-regulation was inhibited by the caspase-3 inhibitor Z-DEVD-fmk and thus involves caspases, as was mentioned previously in other reports (41). On the contrary, Z-VAD-fmk enhanced CD43 down-regulation, presumably via its action on non-caspase enzymes (42). Both caspase inhibitors significantly prevented the scrambling of phospholipids assessed by annexin V binding. We partially confirm the observation of Pryde *et al.* that Z-VAD-fmk delays rather than completely prevents the externalization of PS by using the same apoptosis protocol (19). They reported that Z-VAD-fmk inhibition was maximal after 30 min at 37 °C but was no longer detected after 90 min. In our work, maximal inhibition was observed between 1 and 2 h (60 $\pm$ 14% inhibition of the percentage of annexin⁺ neutrophils). It then decreased, but after a 5 h-incubation at 37 °C a 30% inhibition by Z-VAD-fmk was

still detected (data not shown). Concerning mitochondria, we found that artificial depolarization with cyanide 4-trifluoromethoxyphenylhydrazone (18) during apoptosis did not induce or accelerate the PS externalization or the blebbing phase (data not shown).

PKC inhibition by staurosporine has been reported to inhibit PS externalization in neutrophils incubated overnight at 15 °C and then at 37 °C without preventing apoptosis, as defined by nuclear morphology analysis (19). Because staurosporine is a rather nonspecific inhibitor of protein kinases (43), we used bisindolylmaleimide I (Gö6850), a specific inhibitor of traditional and new (α , β , γ , δ , ϵ , η , and θ) isozymes of PKC and chelerythrine chloride, which blocks all PKC isozymes, including the atypical PKC- ζ (44). Bisindolylmaleimide, chelerythrine, and staurosporine indeed blocked PS exposure but did not prevent the blebbing and even increased further the release of R2/R3 vesicles. CD43 down-regulation during neutrophil apoptosis was not affected by bisindolylmaleimide, chelerythrine, or staurosporine. The PKCs that have been shown to be activated (*i.e.* translocated from the cytosol to the plasma membrane) during neutrophil spontaneous apoptosis are the traditional PKC- β and the new PKC- δ (45). Caspase-3-activated PKC- δ was shown to be directly involved in apoptosis-related nuclear morphology changes and DNA fragmentation (45). Our results indicate that these PKCs would be involved in PS externalization but not in cell blebbing or in the decrease of CD43 expression.

The role of myosin as the motor behind membrane blebbing is suggested by various data; microinjection of active MLCK induces membrane blebs (46), MLCK-inhibitors decrease membrane blebbing, and the phosphorylation of the myosin regulatory light chain is increased in blebbing cells (24). We here confirm this role of MLCK in neutrophils, because the MLCK inhibitors ML9 and ML7 prevented neutrophil blebbing and R2/R3 vesicle release. By contrast, MLCK inhibition had no effect on PS externalization and did not interfere with CD43 down-regulation.

It is worth noting that CD16 (receptor III for the Fc portion of IgG) expression is regulated in a similar way to that of CD43 during neutrophil apoptosis (2, 19). Indeed, we observed that CD16 down-regulation was inhibited by caspase inhibitors but not modified by PKC or MLCK inhibitors (data not shown). The mechanism involved in the decrease of CD43 expression may thus apply to the down-regulation of other membrane receptors.

In conclusion, our data on the three initial membrane effects of neutrophil spontaneous apoptosis, *i.e.* phospholipid flip-flop, membrane blebbing, and decreased CD43 expression, show that these effects result from distinct signaling mechanisms that may occur independently of each other. In the presence of the caspase-3 inhibitor, cell blebbing may occur without PS exposure or decreased CD43 expression. In the presence of PKC inhibitor bisindolylmaleimide, blebbing, R2/R3 vesicle release, and CD43 down-regulation may be observed in the absence of PS externalization. In the presence of the MLCK inhibitor ML9, PS externalization and CD43 down-regulation may occur despite the lack of membrane blebbing. A similar independence of membrane blebbing from other apoptotic events has recently been reported in a T cell line (47).

The observation that ML9 has no effect on CD43 expression, although it efficiently blocks the cell blebbing process, contradicts our initial hypothesis of a decrease in CD43 membrane expression resulting from the release of blebs bearing these receptors. Furthermore, time lapse video sequences show that only a few blebs are released. Similarly, quantification by flow cytometry of R2/R3 vesicles results in a mean of one released bleb per neutrophil during a 2 h period at 37 °C. This cannot

account for the 50% decrease in CD43 expression observed on apoptotic cells during the same incubation period. Moreover, fluorescent and electronic microscopy observations show that membrane blebs are not enriched in CD43 but, on the contrary, that CD43 is almost excluded from these protrusions. Similar segregation of CD43 clusters away from membrane blebs had been observed previously during galectin-1-induced T cell apoptosis (48).

Although we conclude that CD43 down-regulation is independent from the blebbing process, the fact remains that the entire CD43 molecule is released and is most probably inserted in membrane vesicles. Indeed, cell-bound CD43 fragments are not produced, as shown by Western blots produced with the anti-CD43cyto pAb directed against the CD43 whole intracellular domain. The same decrease of cell-associated CD43 was measured by quantitative evaluation of Western blot-scanned bands with the mAb recognizing an extra cellular epitope and with the anti-CD43cyto pAb. Internalization of the molecule was further excluded by flow cytometry analysis of permeabilized and unpermeabilized cells, which gave similar results. In our hands CD43 was never internalized, even after antibody cross-linking or neutrophil stimulation by formyl-methionyl-leucyl-phenylalanine (16, 49).³

Ultracentrifugation of the cell supernatant revealed that CD43 is indeed released via CD43-bearing microparticles. These microparticles, which are ~10-fold smaller than the bleb-derived vesicles analyzed here, were not included in our flow cytometry analysis (Fig. 6) because they were lost in the centrifugation and washing phases. They represent plasma membrane structures because they co-express on their surface annexin-binding phosphatidylserine, membrane receptors such as CD43 and CD11b, and the neutrophil-specific CD66b membrane marker. CD43 density on microparticles is ~10 times that of native neutrophils. The fact that the release of CD43-rich microparticles account, at least in part, for the decrease in neutrophil CD43 expression is further suggested by the observation that both phenomena are inhibited by the caspase-3 inhibitor Z-DEVD-fmk and that none are affected by the MLCK-inhibitor ML9.

Interestingly, these apoptosis-derived microparticles express significant levels of CD63, a tetraspanin present in azurophilic granule membrane and poorly expressed on plasma membrane of native or apoptotic neutrophils (3).⁴ High levels of tetraspanins are considered to be a hallmark of exosomes, microparticles of endocytic origin (50), that distinguish them from microparticles formed directly from the plasma membrane in various cell types (51). This suggests that the origin of apoptosis-derived microparticles differ from that of preformed membrane vesicles, or "ectosomes," which are released during neutrophil activation (52–54) and do not express CD63 (53).

CD43 was partially cleaved during the shedding of microparticles, leading to a 26-kDa membrane-bound fragment. This fragment, the size of which corresponds to the combined intracellular and transmembrane regions of CD43 (259 amino acids) (55), results from a cleavage close to the membrane that would release the whole extracellular portion of the CD43 molecule. This peculiar proteolysis, specifically observed on microparticles but not on apoptotic cells, is reminiscent of the secretion of interleukin-1 by the monocytic cell line THP-1 (56). Indeed, the secretion of a bioactive interleukin-1, despite its lack of a secretory signal sequence, was shown to result from a proteolytic cleavage of the precursor molecule in shed microparticles (56). In the case of CD43, its proteolysis would have the follow-

³ S. Seveau and L. Halbwachs-Mecarelli, unpublished observations.

⁴ P. Nusbaum and L. Halbwachs-Mecarelli, personal observation.

ing consequences: (i) the depletion of an anti-adhesion molecule on the surface of microparticles enhancing putative contacts with other cells; and (ii) the release of a 120-kDa extracellular CD43 fragment (14) analogous to the previously described Galgp plasma protein (57). We can only speculate on the role of a soluble form of CD43 or CD43-bearing microparticles, because CD43 counter-receptors are still mostly unknown. Leukocyte-derived microparticles bearing PSGL-1, a mucin-like molecule similar to CD43, have been described in the blood where they participate in thrombus formation via PSGL-1 binding to platelet P-selectin (58). We are currently analyzing a possible anti-adhesive role of soluble CD43 or CD43-rich microparticles as described previously with cancer-secreted CD43 (59). Such an anti-adhesive function could synergize with the anti-inflammatory effects described recently for the ectosomes released during neutrophil activation (60).

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