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Human Neutrophils Lose Their Surface FcγRIII and Acquire Annexin V Binding Sites During Apoptosis In Vitro

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We have previously reported that neutrophilic granulocytes rapidly release part of their FcγRIII from the plasma membrane upon in vitro activation, probably by proteolytic cleavage. In plasma and other body fluids, released or soluble FcγRIII has been found in considerable amounts. In the present study, neutrophils were kept in maintenance culture for 18 to 24 hours. Forty percent of the neutrophils completely lost FcγRIII, and the remainder of the cells showed a 60% decrease in FcγRIII expression on their surface. Released FcγRIII was detected in the culture supernatant. Nevertheless, more than 90% of the cells was viable as judged by hydrolysis of fluorescein diacetate. The presence of interferon γ, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor, but not interleukin-3 (IL-3), IL-6, or IL-8, in the culture medium increased the number of cells that still expressed FcγRIII. We found that this loss of FcγRIII was not the result of cell activation but correlated strongly with apoptosis. The FcγRIII-negative subpopulation exhibited typical morphologic changes, such as nuclear condensation and DNA fragmentation. Furthermore, this subpopulation appeared to have acquired the property of binding Annexin V, a calcium-dependent, phospholipid-binding protein with high affinity for phosphatidylserine. The external exposure of this phospholipid by cells has been reported to occur during apoptosis. The property of Annexin V binding was not shared by the nonapoptotic, FcγRIII-positive subpopulation. In this respect, we identified binding of Annexin V as an convenient marker for apoptotic cells. Our results indicate that soluble FcγRIII in body fluids might be derived for a large part from neutrophils undergoing apoptosis in the tissues.

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POLYMORPHONUCLEAR neutrophils play an important role in the inflammation process. As a first-line defense, neutrophils leave the circulation upon stimulation and enter the inflamed site to subsequently kill the invading micro-organisms. For this purpose, neutrophils express receptors on their plasma membranes for IgG antibodies and complement fragments present on opsonized micro-organisms. The receptors for the Fc region of IgG (FcγR) present on neutrophils are the transmembrane FcγRIIa and the PI-linked FcγRIIIb. In addition, neutrophils can be induced by certain cytokines to express the high-affinity FcγRI.

In vitro, activated neutrophils shed membrane-bound FcγRIII.[1] Released or soluble (s) FcγRIII derived from neutrophils has been identified in plasma and other body fluids.[2] sFcγRIII levels do not correlate with the number of neutrophils in the peripheral blood and are not affected by changes in the distribution of the neutrophils between the various pools (circulating, marginating, or storage pool).[3] In another study, we analyzed in healthy volunteers the effect of in vivo administration of one single dose of granulocyte colony-stimulating factor (G-CSF; 300 μg, subcutaneously [SC]) on membrane characteristics, eg, the Fcγ receptor profile, of newly generated neutrophils.[4] We observed that the increase in neutrophil count was followed about 1 week later by an increase in sFcγRIII. When we studied the changes in the Fcγ receptor profile of mature neutrophils during culture in vitro, we noticed that FcγRIII was strongly decreased on a subpopulation of cells. This subpopulation was smaller when interferon γ (IFNγ), granulocyte-macrophage–CSF (GM-CSF), or G-CSF was included in the culture medium. It has been described that these cytokines modulate the apoptosis of neutrophils.[5] Hence, we investigated the relationship between the shedding of FcγRIII and apoptosis of neutrophils. The results show that loss of FcγRIII expression closely correlates with apoptosis.

MATERIALS AND METHODS

Antibodies and cytokines. The following fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (MoAbs) were used: CLB irrelevant murine control MoAbs of the IgG1 and IgG2a subclass, CLB-B2.12 (CD11b), CLB mon-gran/2 (CD13), CLB mon/1 (CD14), CLB gran/2 (CD15), 3G8 (CD16), CLB gran-B-ly/1 (CD24), IV.3 (CD32), CLB T200/1 (CD45), CLB-gran/12 (CD63), 22 (CD64), and B13.9 (CD66b). The 3G8, IV.3, and 22 MoAbs were from Medarex (West Lebanon, NH). All other MoAbs were produced in our own laboratory and were clustered during the International Workshops on Leukocyte Differentation Antigens.

rG-CSF was obtained from Amgen (Thousand Oaks, CA), rGM-CSF from Sandoz (Basel, Switzerland), and rIFNγ from Boehringer Ingelheim (Mannheim, Germany).

Annexin V FITC labeling. Annexin V was prepared by cDNA recombinant techniques with plasmid pRH291 and purified as described before.[6] The preparation was more than 99% pure. The only difference detected between recombinant and natural Annexin V was an unblocked N-terminal alanine in recombinant Annexin V, resulting in a slightly higher pI (4.9 *v* 4.8). Annexin V was dialyzed against coupling buffer (50 mmol/L sodium borate/NaOH, pH 9.0, 150 mmol/L NaCl, and 1 mmol/L EDTA). Dialyzed Annexin V (50 μmol/L) was mixed with 50 μmol/L FITC isomer I (F-7250; Sigma, St Louis, MO) and incubated for 2 hours at 37°C. The coupling

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Table 1. Effect of Culturing of Neutrophils on Antigen Expression

	MFI Before Culture	MFI of Positive Cells After Culture	% Positive Cells After Culture*
IgG_1 control	12 ± 7	8 ± 2	100
IgG_{2a} control	9 ± 4	8 ± 2	100
CD11b (IgM)	85 ± 8	36 ± 2	100
CD13 (IgG_{2a})	87 ± 8	64 ± 3	78 ± 2
CD14 (IgG_{2a})	22 ± 2	14 ± 1	100
CD15S (IgM)	502 ± 21	511 ± 38	82 ± 0.4
CD16 (IgG_1)	898 ± 22	367 ± 74	61 ± 2
CD24 (IgG_1)	328 ± 15	364 ± 8	80 ± 3
CD32 (IgG_{2b})	86 ± 13	73 ± 6	77 ± 4
CD45 (IgG_1)	86 ± 14	75 ± 3	100
CD63 (IgG_1)	14 ± 5	15 ± 3	100
CD64 (IgG_1)	14 ± 3	11 ± 2	5 ± 2
CD66b (IgG_1)	32 ± 2	22 ± 3	100

Results shown are means of three experiments ± SD.

Abbreviation: MFI, mean fluorescence intensity.

* Before culture, 100% of the cells were positive for all antigens tested except for CD64 (5% ± 2% positive before culture).

reaction was then stopped by the addition of 100 mmol/L glycine. The mixture was first dialyzed against 50 mmol/L Tris/HCl, pH 8.0, 80 mmol/L NaCl, and 1 mmol/L EDTA and subsequently applied to a Mono Q column (Pharmacia, Uppsala, Sweden). The bound proteins were eluted by an NaCl gradient. The eluted peaks were analyzed for protein contents and absorbance at 492 nm (for FITC $\epsilon^{492} = 78{,}000\ L \cdot mol^{-1}cm^{-1}$). The stoichiometric 1:1 complex was identified and used for the experiments. This FITC labeling did not change the binding capacities as measured by ellipsometry.[7]

Cell isolation and culture. Blood was obtained from healthy volunteers. Granulocytes were purified from the buffy coat of 500 mL of blood anticoagulated with 0.4% (wt/vol) trisodium citrate, pH 7.4, as described.[8] In short, mononuclear cells and platelets were removed by density gradient centrifugation over isotonic Percoll (Pharmacia) with a specific gravity of 1.076 g/mL. Erythrocytes were lysed by short treatment of the pellet fraction with ice-cold isotonic NH_4Cl solution (155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, 0.1 mmol/L EDTA, pH 7.4). The remaining granulocytes were

Table 2. Effect of Cytokines on Fcγ Receptor Profile

	No Additions	100 U/mL IFNγ	10^{-10} mol/L GM-CSF	20 ng/mL G-CSF
% Positive cells after culture				
FcγRI (CD64)	5 ± 2	83 ± 5	5 ± 2	7 ± 3
FcγRII (CD32)	77 ± 4	87 ± 2	78 ± 7	88 ± 4
FcγRIII (CD16)	61 ± 2	77 ± 1	72 ± 5	77 ± 6
MFI of positive cells after culture				
IgG_1 control	8 ± 2	10 ± 1	8 ± 0.1	10 ± 2
IgG_{2a} control	8 ± 2	25 ± 5	13 ± 7	16 ± 9
FcγRI (CD64)	11 ± 2	32 ± 6	11 ± 2	11 ± 1
FcγRII (CD32)	73 ± 6	73 ± 10	72 ± 10	83 ± 8
FcγRIII (CD16)	367 ± 74	340 ± 99	326 ± 78	363 ± 69

Results shown are means of three experiments ± SD. For the percentage of positive cells and MFI before culture, see Table 1.

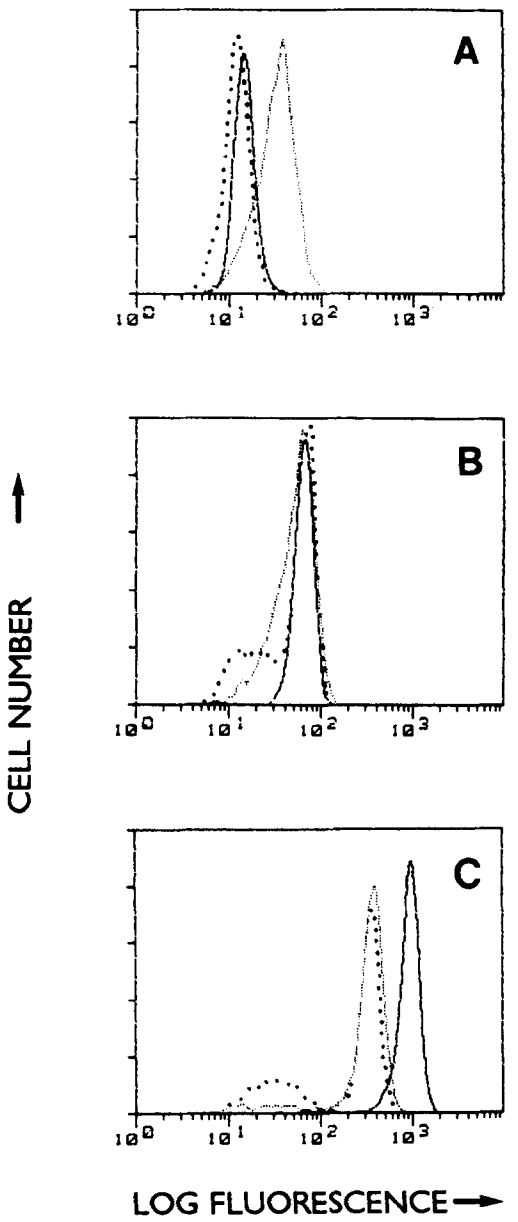

Fig 1. Expression of Fcγ receptors upon culturing of human neutrophils as measured by immunofluorescence in a flow cytometer. (A) Binding of FITC-labeled MoAb 22 (CD64), directed against FcγRI, to neutrophils before and after culturing for 18 hours (——, before culture, · · ·, after culture, without additions; ·····, after culture in the presence of 100 U of IFNγ per milliliter). (B) Binding of FITC-labeled MoAb IV.3 (CD32), directed against FcγRII, to neutrophils before and after culturing for 18 hours (symbols as in [A]; similar results were found in the presence of 20 ng of G-CSF per milliliter). (C) Binding of FITC-labeled MoAb 3G8 (CD16), directed against FcγRIII, to neutrophils before and after culturing for 18 hours (symbols as in [A]; similar results were found in the presence of 20 ng of G-CSF per milliliter or 10^{-10} mol/L GM-CSF). A representative experiment of three is shown.

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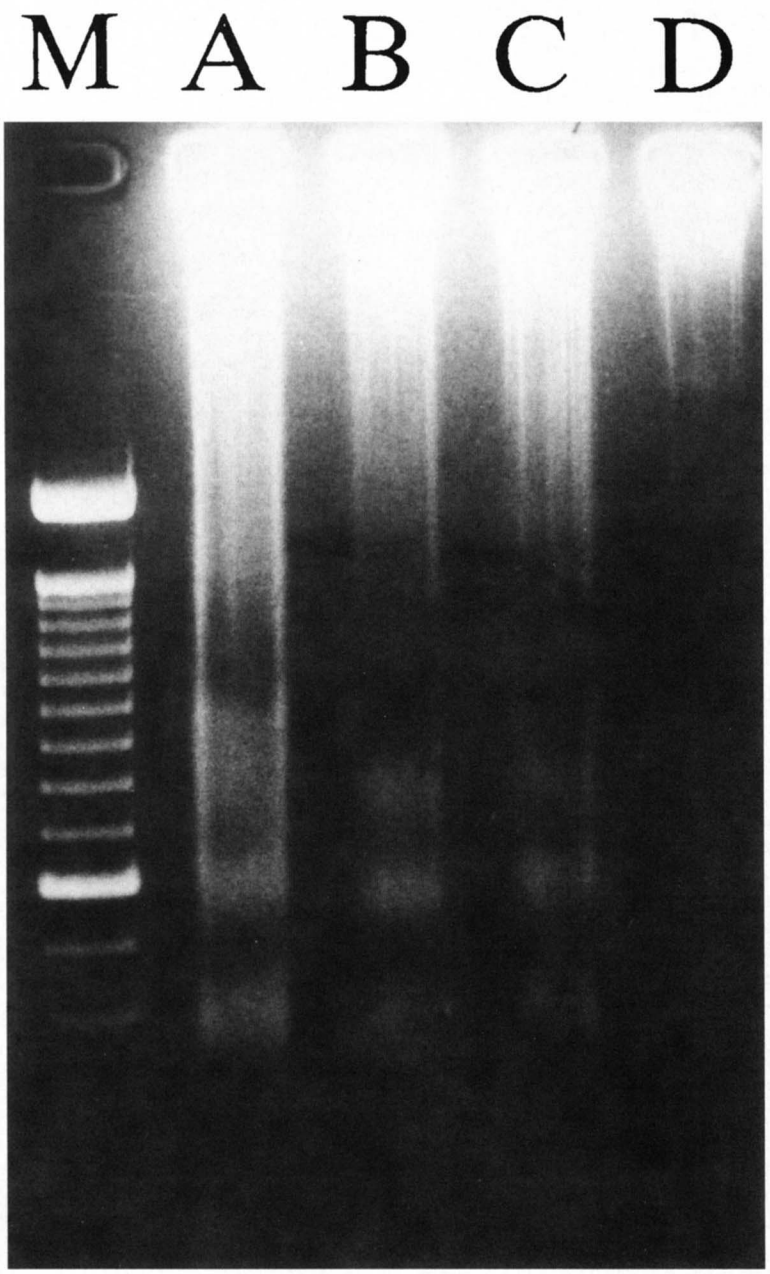

Fig 2. Gel electrophoresis showing DNA fragmentation in neutrophils after culture for 18 hours. DNA from 2×10^6 PMN was added per lane. Lane M, DNA size markers (100-bp difference); lane A, neutrophils cultured for 18 hours in the absence of cytokines; lane B, neutrophils cultured for 18 hours in the presence of IFNγ (100 U/mL); lane C, neutrophils cultured for 18 hours in the presence of GM-CSF (10^{-10} mol/L); lane D, neutrophils cultured for 18 hours in the presence of G-CSF (20 ng/mL). A representative experiment of three is shown.

washed twice in phosphate-buffered saline (PBS) containing human serum albumin (HSA; 0.5%, wt/vol) and were resuspended in culture medium (Iscove's with 10% fetal calf serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, pH 7.4) at a concentration of 10^7 cells/mL. Purity of the granulocytes was greater than 98%, with greater than 95% neutrophils. All cells were viable as determined by fluorescein diacetate (FDA) staining (see below). The suspension was preincubated at 37°C for 15 minutes. Activated cells were removed by cotton-wool filtration and the remaining cells were resuspended in fresh culture medium at a concentration of 10^7 cells/mL. The cells were incubated for 18 hours at 37°C in a humidified atmosphere of 5% CO_2 in 24-well tissue culture plates. Cell viability was determined after culture by FDA staining (see below).

FDA staining. After culture, the granulocytes were washed and resuspended in ice-cold PBS containing 1% (vol/vol) bovine serum albumin (BSA) at a concentration of 10^7 cells/mL. Twenty microliters of this suspension was mixed with 20 μL of FDA (250 ng/mL) and incubated for 30 minutes on ice. After washing, the cells were analyzed in a FACScan flowcytometer (Becton Dickinson, San José, CA). Viable cells take up and hydrolyze FDA, resulting in a green fluorescence signal.

sFcγRIII radioimmunoassay. Soluble FcγRIII was determined by a radioimmunoassay essentially as described before.[2] In short, MoAb CLB FcRgran/1 coupled to CNBr-activated Sepharose 4B was incubated with 200 μL of culture supernatant for 16 hours at room temperature. Each test was performed in triplicate. After washing, the beads were incubated for 5 hours at room temperature with ^{125}I-labeled FcγRIII MoAb BW209/2 (Dr R. Kurrle, Behringwerke

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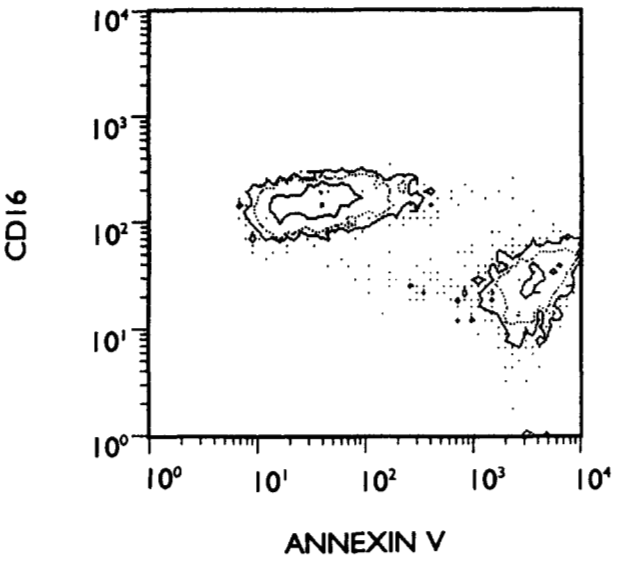

Fig 3. Flow cytometric analysis of human neutrophils after culture for 18 hours. Cells were incubated with biotin-labeled 3G8 (CD16), an MoAb against FcγRIII, and FITC-labeled Annexin V, a protein that binds to phosphatidylserine. A representative experiment of three is shown.

AG, Marburg, Germany). Subsequently, the beads were washed again five times and the bound radioactivity was counted in a gamma counter. A calibration curve was made from serial dilutions of pooled plasma, and the value of the sFcγRIII in the culture supernatant was expressed as a percentage of the amount of sFcγRIII in the normal plasma pool. This last amount was arbitrarily set at 100 U.

Immunofluorescence. Neutrophils were washed and resuspended in ice-cold PBS containing 1% (vol/vol) BSA at a concentration of 10^7 cells/mL. Twenty microliters of this suspension was incubated for 30 minutes with 20 μL of the FITC-labeled MoAbs in the appropriate dilutions. The cells were washed once and analyzed in a FACScan flowcytometer (Becton Dickinson). All incubations and washing steps were performed at 4°C. In contrast with the antibody incubations, incubations with Annexin V and subsequent washings were performed with cells in HEPES buffer (20 mmol/L HEPES, 132 mmol/L NaCl, 6 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 1.2 mmol/L potassium phosphate, 5.5 mmol/L glucose, and 0.5% [wt/vol] HSA, pH 7.4). After washing, these cells were also analyzed by flowcytometry. Maximal and half-maximal binding of annexin V to cultured neutrophils was reached at Ca^{2+} concentrations of 1.5 mmol/L and 0.30 mmol/L, respectively.

DNA fragmentation. DNA fragmentation was analyzed as previously described.[9] Portions of 10^6 neutrophils were washed twice in PBS at 4°C and were resuspended in 20 μL of a solution containing 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.0) containing 0.5% (wt/vol) sodium lauryl sarcosinate and 0.5 mg proteinase K per milliliter. This lysate was incubated for 1 hour at 50°C. Ten microliters of 0.5 mg/mL RNase A was then added to each sample and the incubation was continued for another 1 hour at 50°C. The samples were heated to 70°C and mixed with 10 μL of 70°C sample buffer (10 mmol/L EDTA, 1% [wt/vol] low-gelling-temperature agarose, 0.25% [wt/vol] bromophenol blue, and 40% [wt/vol] sucrose, pH 8.0) before loading into the dry wells of a 2% agarose gel containing 0.5 μg of ethidium bromide per milliliter. Electrophoresis was performed in TAE-buffer (0.04 mol/L Tris/acetate, 2 mmol/L EDTA, pH 8.0) until the marker dye had migrated 5 to 6 cm.

Cell sorting. Cultured neutrophils were washed and resuspended in ice-cold PBS containing 1% (vol/vol) BSA at a concentration of 10^7 cells/mL. This suspension was mixed with an equal volume of FITC-labeled CD16 MoAb 3G8 and incubated for 30 minutes on ice. The cells were then washed, resuspended in PBS/BSA, and stored on ice.

Alternatively, when neutrophils had to be sorted after Annexin V binding, the cells were resuspended in ice-cold HEPES buffer (see Immunofluorescence) at a concentration of 10^7 cells/mL and were incubated with FITC-labeled Annexin V for 30 minutes on ice. The cells were then washed, resuspended in HEPES buffer, and stored on ice.

Cells were sorted by a FACStar+ (Becton Dickinson). During and after sorting, the cells were kept at 4°C. Cytocentrifuge prepara-

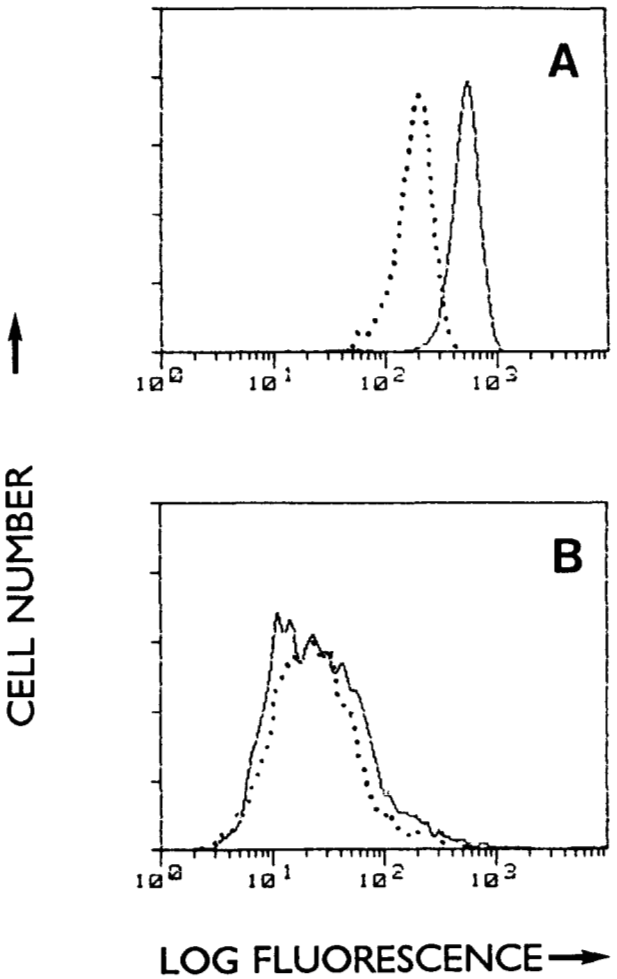

Fig 4. Effect of activation of fresh neutrophils on FcγRIII expression and Annexin V binding as measured by immunofluorescence in a flow cytometer. (A) Binding of FITC-labeled MoAb 3G8 (CD16), directed against FcγRIII, to neutrophils before and after activation with cytochalasin B (5 μg/mL) plus 1 μmol/L fMLP (——, resting neutrophils; ···, activated neutrophils). (B) Binding of FITC-labeled Annexin V, a protein that binds to phosphatidylserine, before and after activation with cytochalasin B (5 μg/mL) plus 1 μmol/L fMLP (symbols as in [A]). With PAF (1 μmol/L) + fMLP (1 μmol/L) or with PMA (100 ng/mL) as activating agents, similar results were obtained. A representative experiment of three is shown.

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tions of the cells after sorting were stained with May-Grünwald-Giemsa. DNA fragmentation was assessed as described above.

RESULTS

Antigen profile of cultured neutrophils. To investigate the in vitro effects of cytokines on the antigen expression of neutrophils, we set up a culture system in which 70% to 80% of the cells are recovered after 18 to 24 hours, with 80% to 90% viability of the recovered cells. Table 1 shows the effects of this culture on the antigen expression of neutrophils. The most pronounced effect was found on the expression of phosphoinositol (PI)-linked FcγRIII (CD16): 40% of the cells lost all surface FcγRIII, whereas on the remaining 60% of the cells the FcγRIII expression decreased to 40% of the value before culture (Fig 1 and Tables 1 and 2). The expression of other surface proteins, either PI-linked or transmembrane, decreased much less and was often restricted to a small percentage of the cells (Table 1). The loss of FcγRIII was not the result of cell activation, because the surface expression of CD63 (a marker of the azurophilic granules) or CD66b (a marker of the specific granules) was not increased (Table 1). There were also no detectable amounts of enzyme from the granules released into the medium (data not shown). sFcγRIII was found in the culture supernatant to the extent of about 50% of the amount found after treatment of neutrophils with PI-specific phospholipase C (ie, 52 ± 13 arbitrary units/10^6 cells [n = 3] with PI-PLC and 28 ± 3 arbitrary units/10^6 cells [n = 10] during culture; mean ± SEM).

We next investigated the effect of cytokine addition to the culture. For the three Fcγ receptors the effects are shown in Table 2. FcγRI, not present on freshly isolated neutrophils, was induced on 80% of the cells when IFNγ (100 U/mL) was included in the culture medium. The percentage of cells that lost their FcγRII expression was diminished by addition of IFNγ or G-CSF (20 ng/mL). IFNγ, G-CSF, and GM-CSF (10^{-10} mol/L) increased by 20% to 25% the percentage of neutrophils that still expressed FcγRIII, but did not affect the expression of FcγRIII per cell. The same cytokines inhibited the appearance of sFcγRIII in the culture supernatant by about 30%. Interleukin-3 (IL-3), IL-6, and IL-8 had no effect on any of these changes.

IFNγ, G-CSF, and GM-CSF prevent DNA fragmentation. It has been described that IFNγ and GM-CSF can modulate the apoptosis of neutrophils.[5] Indeed, in neutrophil suspensions cultured in the presence of IFNγ (100 U/mL), G-CSF (20 ng/mL), or GM-CSF (10^{-10} mol/L), less cells exhibited features characteristic of apoptotic cells, ie, nuclear condensation, cell shrinkage, and cytoplasmic vacuolization. We therefore investigated whether DNA fragmentation was influenced by the presence of these cytokines. As shown in Fig 2, these cytokines clearly decreased the extent of DNA fragmentation.

Cells lacking FcγRIII expression bind Annexin V to their membrane. Phosphatidylserine (PS), which is normally only present on the inner leaflet of the plasma membrane of cells, is expressed on the outer leaflet of the membrane of apoptotic cells.[10] Annexin V is a protein that can bind to phospholipids, and especially to PS, in a calcium-dependent

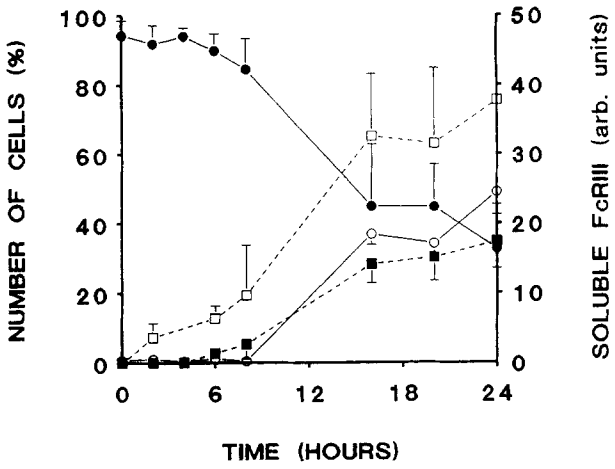

Fig 5. Analysis of cells cultured for different periods ([●] number of cells that still express CD16; [○] number of cells that can bind Annexin V; [■] number of cells with morphologic features of apoptosis [nuclear condensation, vacuolization] enumerated by microscopic counting; [□] concentration of soluble FcγRIII in the culture supernatant). Results shown are means of four experiments ± SD.

fashion.[11] We examined by means of double-color FACS analysis whether cells that had lost their surface FcγRIII bound Annexin V. Binding of Annexin V to 37% ± 0.8% (n = 3) of neutrophils was observed after culture for 18 hours. These Annexin V-binding cells appeared to have lost their surface FcγRIII because there were no double-colored events after coincubation with MoAb 3G8 (Fig 3). The percentage of FcγRIII-positive cells, 59% ± 4.7% (n = 3), was not changed by the coincubation with Annexin V. IFNγ, G-CSF, and GM-CSF diminished the number of Annexin V-binding cells and increased the number of FcγRIII-positive cells (data not shown). Also, after culturing in the presence of cytokines, only FcγRIII-negative neutrophils bound Annexin V. On the other hand, neutrophils activated by short-term treatment with either 1 μmol/L platelet-activating factor (PAF) plus 1 μmol/L fMLP, 100 ng/mL of PMA, or 5 μg/mL cytochalasin B plus 1 μmol/L fMLP, lost part of their surface FcγRIII, but did not bind Annexin V (Fig 4).

Loss of FcγRIII expression and acquirement of Annexin V binding sites correlate in time. To show the time dependency of the different features, we cultured neutrophils for various periods and examined FcγRIII expression, Annexin V binding, cell morphology, and soluble FcγRIII levels. As shown in Fig 5, changes in the first three features correlated in time, whereas the amount of soluble receptor already slowly increased in the first 8 hours. During this period, the expression of FcγRIII on the cells decreased in MFI from 2,191 ± 494 to 1,453 ± 229. Thus, it seems that the complete loss of FcγRIII expression, acquirement of Annexin V binding sites, and nuclear condensation occur simultaneously.

Expression of FcγRIII and binding of Annexin V serve as markers for neutrophil apoptosis. To unequivocally show that neutrophils that have lost their FcγRIII indeed exhibit features of apoptosis, we sorted cultured cells on FcγRIII

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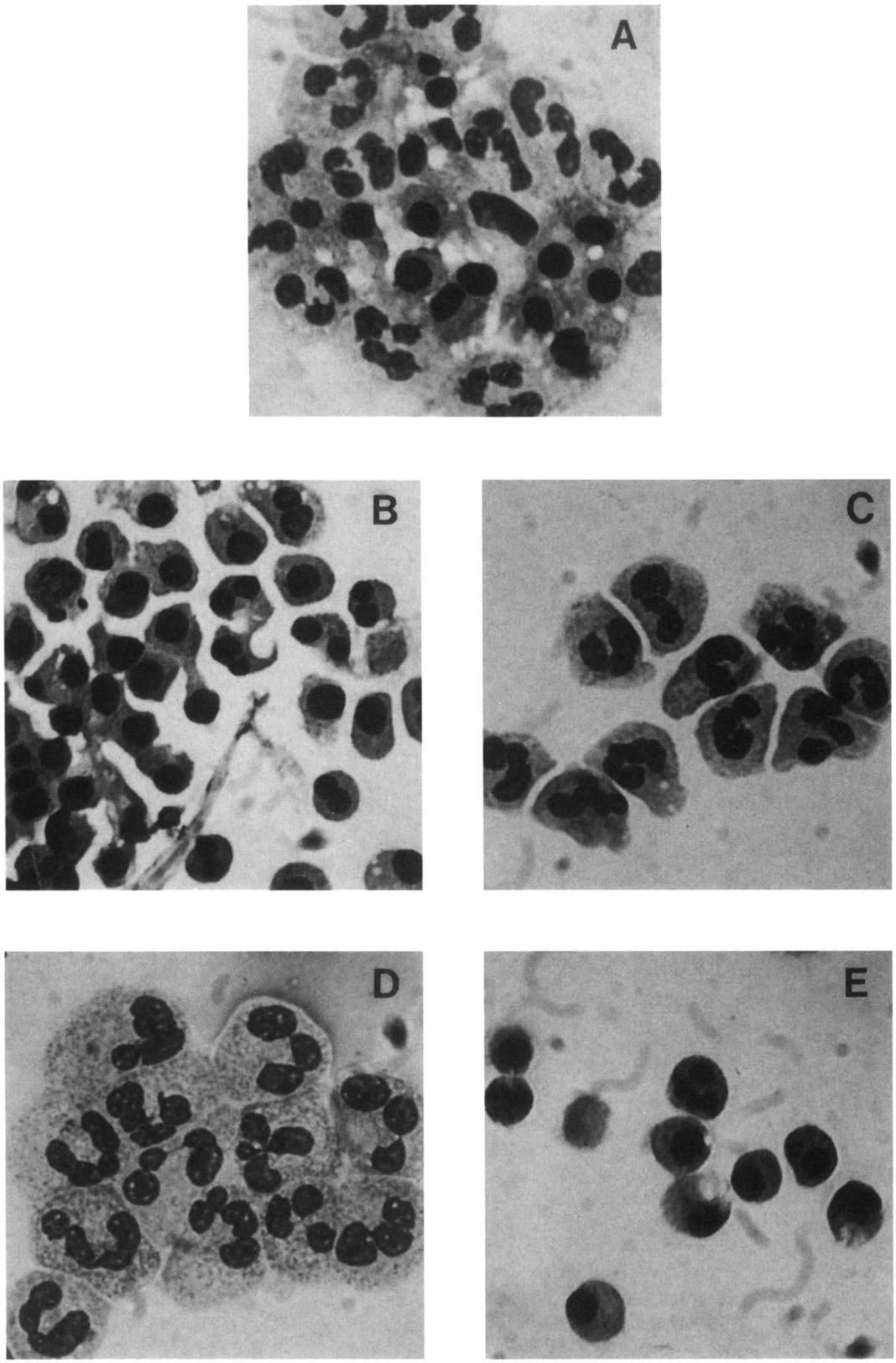

Fig 6. Morphologic features of neutrophils cultured for 18 hours. (A) Cells before sorting. (B) Cells after sorting; no FcγRIII expression. (C) Cells after sorting; with FcγRIII expression. (D) Cells after sorting; no Annexin V binding. (E) Cells after sorting; Annexin V binding.

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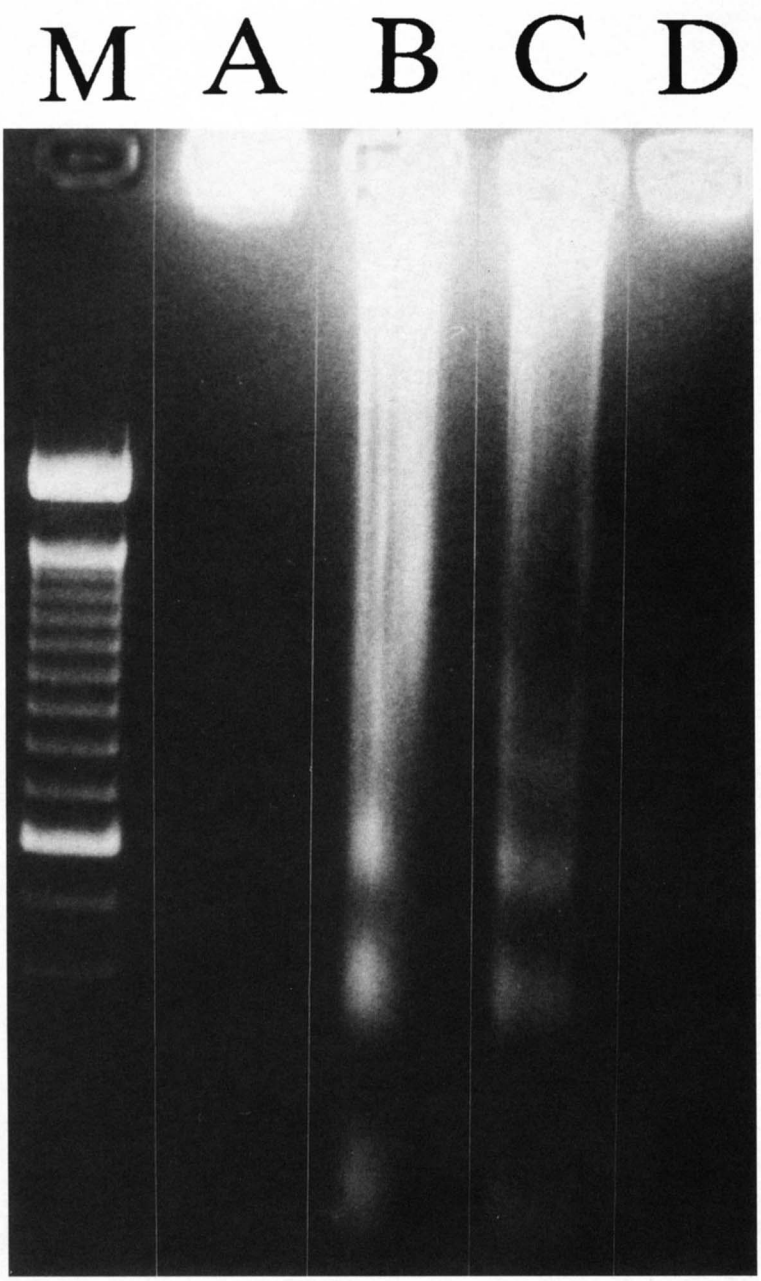

Fig 7. Gel electrophoresis demonstrating DNA fragmentation in neutrophils after culture for 18 hours after sorting. DNA from 1 × 10^6 PMN was added per lane. Lane M, DNA size markers (100-bp difference). Lane A, cells sorted on FcγRIII expression; FcγRIII positive. Lane B, cells sorted on FcγRIII expression; FcγRIII negative. Lane C, cells sorted on Annexin V binding; Annexin V positive. Lane D, cells sorted on Annexin V binding; Annexin V negative. A representative experiment of three is shown.

expression. Normal morphology (Fig 6) without DNA fragmentation (Fig 7) was found in those cells that still expressed FcγRIII. In contrast, cells that had lost FcγRIII expression showed nuclear condensation (Fig 6) and DNA fragmentation (Fig 7). When binding of Annexin V was used as the selection criterion for cell sorting, the results depicted in Figs 6 and 7 confirmed that Annexin V-binding cells were apoptotic. Taken together, these results clearly show that apoptotic neutrophils lose their surface FcγRIII expression and acquire binding sites for Annexin V. In contrast, non-apoptotic neutrophils, either quiescent or activated, do not expose Annexin V binding sites.

DISCUSSION

Apoptosis of neutrophils occurs spontaneously during long-term in vitro culturing.[5,12] A prolonged survival can be achieved by addition of the cytokines IFNγ, GM-CSF, or G-CSF to the culture medium.[5,13,14] Moreover, it has been shown that IFNγ[5] and GM-CSF[5,14] prevent the occurrence of neutrophil apoptosis in vitro, whereas conflicting data have been reported on the role of G-CSF[5,14] and IL-6[14,15] in this process. IL-3 and IL-8 do not have any influence.[14] GM-CSF and G-CSF play an important role in granulocytopoiesis.[16,17] Moreover, we have shown that G-CSF in vivo equips the newly generated neutrophils with the high-affinity IgG

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receptor FcγRI.[4] IFNγ also induces the expression of FcγRI, both in vivo[18,19] and in vitro.[20] However, cytokines not only influence the cell phenotypically,[4,21] but also functionally with respect to antibody-dependent cellular cytotoxicity[22] and priming for respiratory burst activity.[23] Elevated levels of G-CSF have been found during bacterial infections.[24] Thus, these cytokines play a complex role in the inflammation process; they increase the lifetime of neutrophils and prepare newly generated cells for a strong functional response.

In the present study, we observed a dramatic decrease of surface FcγRIII expression on human neutrophils during in vitro culture, apparently due to shedding of this receptor. Addition of IFNγ, GM-CSF, or G-CSF to the culture diminished this loss. Moreover, less sFcγRIII was found in the culture supernatants when cytokines were added. In kinetic experiments, we found that the appearance of sFcγRIII in the culture supernatants preceded the complete loss of FcγRIII expression from the neutrophils' surface. However, the expression of FcγRIII on the cells already decreased by about 30% in the first 8 hours of culture, which probably explains the increased levels of sFcγRIII in the culture supernatants during this period. The complete loss of surface FcγRIII was restricted to apoptotic neutrophils, as judged by morphologic criteria and DNA fragmentation. After in vivo administration of a single dose of G-CSF to healthy volunteers, an increase in the neutrophil count was followed about 1 week later by elevated levels of sFcγRIII.[4] These results indicate that the level of sFcγRIII in body fluids may be derived for a large part from neutrophils undergoing apoptosis in the tissues.

Our results show that the plasma membrane of the neutrophil undergoes specific structural changes during apoptosis, ie, it loses FcγRIII expression and acquires Annexin V binding sites. These changes occur during a phase of apoptosis in which the neutrophil still maintains its cellular integrity. Macrophages recognize elements of the changed membrane organization and phagocytose the apoptotic cell before it will release its inflammatory constituents into the environment through lysis.[10] Amino sugars and amino acids have been shown to inhibit the macrophage uptake of apoptotic neutrophils in a charge-dependent manner.[25] In this light, it has been proposed that the apoptotic cell bears a particular anionic group that may be masked by cationic structures in the preapoptotic stage.[25] The release of surface FcγRIII, which may arise from a proteolytic process involving a protease[2] in analogy with the induction of the CED3 protein during programmed cell death of *Caenorhabditis elegans,*[26] results in a residual membrane-associated fragment with a negatively charged character.[2]

Annexin V is a member of a family of proteins that are structurally related and exhibit Ca^{2+}-dependent phospholipid-binding properties.[11] Annexin V can bind to various phospholipid species and shows it highest affinity for phosphatidylserine.[7] Fadok et al[10,27] recently reported the appearance of phosphatidylserine on the surface of apoptotic lymphocytes and neutrophils, which is recognized by a specific receptor on macrophages. These investigators showed the exposure of phosphatidylserine functionally by a test in which apoptotic cells reduced the clotting time. Phosphatidylserine is appreciated as a catalyst that accelerates coagulation reactions of the vitamin K-dependent coagulation factors. Annexin V has been shown to inhibit coagulation reactions by binding to the catalytic phospholipids.[28,29] Together with the present work, it is conceivable but still inconclusive that the Annexin V binding site on the apoptotic neutrophil is phosphatidylserine. As indicated above, Annexin V is able to bind to other phospholipid species and, furthermore, it can also bind to proteins, as has been shown for actin.[30] The structural nature of the Annexin V binding site has to be further explored, just like the question of whether this site is involved in the recognition of the apoptotic cell by macrophages.

In conclusion, our data show that neutrophils undergo marked phenotypical changes during the process of apoptosis in vitro. The loss of FcγRIII and the expression of Annexin V binding sites are convenient markers to follow this process.

NOTE ADDED IN PROOF

After the acceptance of this manuscript, a report was published by Dransfield et al reporting similar loss of FcγRIII expression during in vitro apoptosis of human neutrophils (*J Immunol* 153:1254, 1994).

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