Incubation of neutrophils with cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF) delays their loss of function and changes in cellular morphology that are characteristic of apoptosis. Adenosine triphosphate (ATP) and the diadenosine polyphosphates Ap₄A and Ap₃A were almost as effective as GM-CSF in delaying neutrophil apoptosis. The nucleotides could thus preserve cellular morphology, protect against chromatin fragmentation, and preserve functions such as NADPH oxidase activity and expression of CD16. Moreover, addition of ATP, Ap₃A, and Ap₄A together with GM-CSF resulted in more pronounced protection from apoptosis than was observed during incubation with either the cytokine or the nucleotides alone. Because ATP, Ap₃A, and Ap₄A may be secreted from activated platelets, these observations suggest that platelet-derived products, perhaps acting in combination with endothelial-derived or immune cell-derived cytokines, can regulate neutrophil function during certain types of inflammation.

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MATERIALS AND METHODS

Materials. Ap₄A, Ap₃A, ATP, adenosine, adenosine deaminase, fMet-Leu-Phe, PMA, diphenylamine, cytchrome c, lulinol, perchloric acid, Triton X-100, RNAase, sarkosyl, and proteinase K were from Sigma (Poole, UK); trichloroacetic acid (TCA) was from BDH (Lutterworth, UK). Recombinant (r)GM-CSF (97% purity) was a non-glycosylated peptide from Glaxo (Greenford, UK) and had an activity of 1.5 µmol/mg protein in the AML-199 proliferation assay. Stock solutions of fMet-Leu-Phe and PMA were freshly made up in DMSO and the corresponding amounts of solvent were added to controls.

Preparation of neutrophils. Neutrophils were isolated from heparinized human blood from healthy volunteers by one step centrifugation through neutrophil-isolating medium (NIM; Cardinal Associates, Santa Fe, NM) as described in the manufacturer's instructions. After hypotonic lysis to remove contaminating erythrocytes, neutrophils were suspended in RPMI-1640 medium (Flow Laboratories, Rickmansworth, UK) supplemented with 2.5% fetal calf serum (Sigma) and 2 mmol/L L-glutamine (Flow Laboratories) and then counted using a Fuchs-Rosenthal haemocytometer slide. Cell viability (>95%) and cell purity (>97%) after purification were routinely determined by Trypan blue exclusion and Wright's staining, respectively.

Culture of neutrophils. Neutrophils (5 × 10⁶/mL) in RPMI 1640 supplemented with 2.5% fetal calf serum and 2 mmol/L L-glutamine were incubated in polypropylene conical tubes at 37°C in the absence (control) or presence of ATP, Ap₃A, Ap₄A, or adenosine (all at 50 µmol/L), added in the presence or absence of rGM-CSF (50 U/mL).
Some incubations also contained adenosine deaminase (1 U/mL) as indicated. At various incubation times aliquots were removed and processed as described below.

Survival and apoptosis. Aliquots of neutrophils were mixed with 0.1% Trypan blue, incubated for 3 minutes, and the number of viable and nonviable neutrophils counted. Survival was expressed as the percentage of neutrophils remaining viable (ie, those that excluded Trypan blue) of the total number in the original suspension.

For morphologic estimation of apoptosis, 10^5 neutrophils were cytocentrifuged, fixed and stained with May-Grünewald-Giemsa (Sigma), air dried, and then examined microscopically. A minimum of 800 cells per cytospin were counted and the number of apoptotic cells was expressed as percentage of the total cells on the slide.

**NADPH oxidase activity.** Chemiluminescence was assayed in a reaction mixture containing equal numbers of viable (ie, Trypan blue-excluding) neutrophils and 10 μm/L luminol. After the addition of the stimuli (Met-Leu-Phε at 1 μm/L and PMA at 0.1 μg/mL), photon emission was measured using an LKB Wallac 1251 luminometer (LKB Wallac, Turku, Finland) in a final volume of 1 mL.**20** Superoxide secretion was monitored by determination of superoxide dismutase-inhibitable reduction of cytochrome c**29,30** in a reaction mixture containing equal numbers of viable neutrophils and 75 μm/L cytochrome c. After the addition of stimuli, absorbance increases at 550 nm were measured using a Perkin Elmer Lambda 5 spectrophotometer in a final volume of 1 mL. Reference cuvettes additionally contained 30 μg/mL superoxide dismutase.

**Chromatin structure.** Quantitation of low molecular weight DNA was carried out as described previously.**3** Briefly, 2.5 × 10^6 neutrophils were centrifuged in microfuge tubes at 13,000g for 2 minutes, washed with cold phosphate-buffered saline (PBS) (10 mmol/L potassium phosphate, 0.9% NaCl, pH 7.4) and then lysed with 10 mmol/L Tris, pH 7.5, 1 mmol/L EDTA, and 0.2% Triton X-100. After 15 minutes of incubation on ice, low and high molecular weight DNA were separated by centrifugation at 13,000g at 4°C for 20 minutes. Centrifugation-resistant low molecular weight DNA in the supernatant was transferred to separate tubes and precipitated overnight at 4°C with 12.5% trichloroacetic acid (TCA). Cold TCA (12.5%) was also added to the pellets, which were then left overnight at 4°C. Samples were then centrifuged at 13,000g at 4°C for 7 minutes, and DNA in the precipitates was extracted with 30 μL of 5 mmol/L NaOH and 30 μL of 1 mol/L perchloric acid at 70°C for 20 minutes. Then, 120 μL diphenylamine reagent**1** was added to each sample and incubated overnight at 37°C. One hundred twenty microcilters from each sample was then transferred to a well of a flat-bottomed 96-well plate and the absorbance at 600 nm was measured using a Bio-Rad 3550 plate reader.

The extraction and electrophoresis of fragmented DNA was assessed using a previously described method**6** with some modifications. Briefly, 5 × 10^6 neutrophils were washed, lysed, then centrifuged at 13,000g at 4°C for 20 minutes exactly as described above.

Low molecular weight DNA in the supernatant was transferred to separate microfuge tubes, mixed with 20 μg/mL RNase, and incubated for 1 hour at 37°C. Added to the pellets was 0.5 mL of 50 mmol/L Tris, 10 mmol/L EDTA, 0.5% Sarcosyl, and 0.5 μg/mL proteinase K; this was incubated overnight at 48°C. Low and high molecular weight DNA were then extracted twice with 1 volume phenol/chloroform/iso-amyl alcohol (25:24:1, respectively) and once with chloroform/iso-amyl alcohol (24:1). DNA in the extracts was then precipitated with 0.5 mol/L NaCl and 1 volume isopropanol for 18 hours at −20°C. The samples were then centrifuged for 10 minutes at 13,000g, and 200 μL of 70% ethanol was gently added to the precipitate; the samples were recentrifuged for 2 minutes at 13,000g, air dried, and resuspended in water. Three microliters loading buffer (2.5% ficoll, 0.025% bromophenol blue, and 0.025% xylenol cyanol) was added to each sample; this was heated at 75°C for 5 minutes, snap-cooled, and then electrophoresed along with DNA markers on a 1% agarose gel containing 1 μg/mL ethidium bromide at 30 V in Tris-acetate buffer. DNA was visualized under UV and then photographed.

**Receptor expression.** Expression of CD16 (FcγRIII) was measured by FACS analysis using a standard indirect immunofluorescence technique as described previously.**2,3** Cells were suspended in PBS/1% bovine serum albumin (BSA) (globulin free)/0.1% sodium azide, pH 7.2, and incubated with the monoclonal antibody Leu11a (Becton Dickinson, Cowley, UK) as a first layer antibody. FITC-labeled goat-antimouse immunoglobulin was used as a second layer antibody. Both were used at saturating concentrations and nonimmune mouse IgG of the appropriate subclass was used as a specific first layer control. Stained cells were fixed in 1% paraformaldehyde in PBS and were analyzed using a Becton Dickinson Ortho Diagnostics Cyantr Cytron analyzer. Fluorescence distributions represent a total of 5,000 gated events.

**Propidium iodide/CD16 dual labeling.** Dual labeling of neutrophils with propidium iodide (to measure chromatin structure) and anti-CD16 antibodies (to detect FcγRIII expression) was carried out as described previously,**12,23** with some modifications. Briefly, neutrophils were labeled with first (Leu11a) and second layer antibodies as described above. Cells were then washed twice with cold PBS/1% BSA/0.1% azide and fixed for 15 minutes in ice-cold 70% ethanol. The cells were then washed and the pellet suspended in propidium iodide solution (2 μg/mL in PBS) and incubated for 1 hour in the dark at 4°C before cytometric analysis.

**Statistical analysis.** The paired Student’s t-test was used to evaluate the significance of differences between the sample means. Statistical significance was defined at P ≤ .05.

**RESULTS**

**Effects of ATP, ApA, and ApA on neutrophil survival.** Neutrophil apoptosis can be assessed by various parameters, including changes in cellular morphology. Thus, apoptotic neutrophils have a condensed nucleus, condensed cytoplasm, and decreased cell size. Neutrophil suspensions incubated for 24 hours in the absence of any exogenous addition were 75% apoptotic (±10%, n = 9) by these criteria. Addition of Ap4A, Ap2A, or ATP (Fig 1) caused a slight but significant (P > .05) protection against apoptosis, with values of 68% (±11%, n = 9), 68% (±14%, n = 7), and 71% (±5%, n = 5), respectively, when analyzed in a paired Student’s t-test. Addition of GM-CSF alone likewise protected against apoptosis (56% ± 13%, n = 21), but addition of nucleotide ± GM-CSF together resulted in much better protection than was observed with either nucleotide or cytokine alone.

**Effects on DNA fragmentation.** When neutrophils undergo apoptosis, their chromatin breaks down and becomes highly fragmented. This can be detected as either an increased formation of low molecular weight DNA (which can be quantified) or as a DNA ‘‘ladder’’ of nucleosome-sized (180-200 bp) fragments after gel electrophoresis. High and low molecular weight DNA was thus quantified after incubation of neutrophils for 24 hours in culture. In control (untreated) suspensions, 59% (±5%, n = 5) of the DNA was fragmented into low molecular weight (Fig 2). In suspensions treated with either GM-CSF, ApA, or ATP, significantly less (P < .05) DNA was fragmented, with the three agents possessing near equal potency in protecting against DNA fragmentation. However, in suspensions incubated with either ATP or ApA together with GM-CSF, signifi-
Trypan blue exclusion, nuclear condensation, cell size, and cell shape, apoptosis. Neutrophils were incubated for 24 hours, as described in Materials and Methods, and cellular morphology was assessed by Trypan blue exclusion, nuclear condensation, cell size, and cell shape, as described in reference 8. The number of apoptotic neutrophils present after 24 hours is expressed as a percentage of the number of cells counted. (■) control; (□) nucleotide alone; (△) GM-CSF alone; (●) nucleotide + GM-CSF. Bars indicate mean values ± SD. Number of separate experiments: Ap₄A, n = 9; Ap₃A, n = 5; ATP, n = 7.

Fig 1. Effects of GM-CSF, Ap₄A, Ap₃A, and ATP on neutrophil apoptosis. Neutrophils were incubated for 24 hours, as described in Materials and Methods, and cellular morphology was assessed by Trypan blue exclusion, nuclear condensation, cell size, and cell shape, as described in reference 8. The number of apoptotic neutrophils present after 24 hours is expressed as a percentage of the number of cells counted. (■) control; (□) nucleotide alone; (△) GM-CSF alone; (●) nucleotide + GM-CSF. Bars indicate mean values ± SD. Number of separate experiments: Ap₄A, n = 9; Ap₃A, n = 5; ATP, n = 7.

Significantly greater protection against chromatin breakdown was observed compared with the effect of either compound alone (P < .05). Thus, the nucleotides and GM-CSF appeared to have an additive effect on protection against DNA fragmentation. Similar results were observed when DNA fragmentation was analyzed by gel electrophoresis (data not shown). Incubation with GM-CSF, ATP, and Ap₄A resulted in decreased DNA fragmentation, but far lower levels of fragmentation were observed when either ATP or Ap₄A were used in combination with GM-CSF.

Effects of NADPH oxidase activity. As neutrophils age in culture, their ability to generate reactive oxidants via the NADPH oxidase declines. GM-CSF treatment both primes the respiratory burst and protects against this decline in oxidase activity. Incubation of neutrophils for 2 hours with GM-CSF primed luminol chemiluminescence generated in response to stimulation by fMet-Leu-Phe (Fig 3). Under these conditions, the responses of primed neutrophils were 4.8-fold (±1.6, n = 15) greater than in control cells. However, neither ATP, Ap₄A, nor Ap₃A primed fMet-Leu-Phe stimulated oxidase activity after 2 hours of incubation. Instead, the responses of neutrophils incubated for 2 hours with these nucleotides was decreased compared with controls. In addition, in suspensions incubated with ATP plus GM-CSF for 2 hours, oxidase activity was decreased compared with that observed in cultures incubated with GM-CSF alone. However, this decrease in the GM-CSF primed response was not observed in cultures containing Ap₄A + GM-CSF or Ap₃A + GM-CSF at 2 hours. All three nucleotides are unstable in biologic solutions, but the diadenosine polyphosphates are more stable than ATP. It has previously been shown that the nucleotide breakdown product, adenosine, can inhibit some neutrophil responses. Thus, we tested the effects of adenosine on oxidase activity in these experiments. Indeed, incubation of cells for 2 hours with adenosine inhibited both the control oxidase activity and the GM-CSF primed response (Fig 3D). The effects of adenosine on oxidase activity were very rapid. When adenosine was added to control suspensions 1 minute before the addition of fMet-Leu-Phe, oxidase activity was inhibited by 60% (±19%, n = 5). Similarly, when added to primed suspensions (GM-CSF for 2 hours) 1 minute before fMet-Leu-Phe stimulation, the response was inhibited by 89% (±4%, n = 5). However, the addition of adenosine deaminase (1 U/mL) before the addition of adenosine prevented this inhibitory effect on oxidase activity (Fig 4A).

When oxidase activity was measured after 24 hours of incubation, quite different results were obtained. In control suspensions (Fig 3, which shows results from a series of experiments where n = 5), oxidase activity had decreased...
Fig 3. Effects of GM-CSF, Ap4A, Ap3A, adenosine, and ATP on NADPH oxidase activity. Neutrophils were incubated for 2 hours (●) or 24 hours (□) as described in Materials and Methods. NADPH oxidase activity was measured by luminol chemiluminescence after addition of 10 μmol/L luminol and stimulation by 1 μmol/L fMet-Leu-Phe. Values presented are means ± SD (n = 5 experiments); an asterisk (*) represents values significantly different from controls and a dagger (†) significantly different from values obtained in cultures containing either GM-CSF alone or nucleotide alone. All suspensions contained equal numbers of Trypan blue-excluding cells.

Effects on CD16 expression. Previous investigations have shown that there is a link between neutrophil apoptosis and expression of CD16, the low affinity receptor for IgG-containing immune complexes. Functionally active neutrophils express high levels of this receptor, while apoptotic neutrophils are CD16−. Freshly isolated neutrophils express high levels of CD16, but after 24 hours of incubation in culture, only about 6% (±3%, n = 6) of the population expressed this receptor (data not shown). The levels of fluorescence observed in the CD16− population were equivalent to those observed when cells were stained with nonimmune first layer antibody. When neutrophils were incubated for 24 hours with either GM-CSF, ATP, Ap4A, or Ap3A, there was a significant increase in the population of CD16+ cells (25% ± 8%; 16% ± 6%; 15% ± 6%; 15% ± 5%, respectively). Furthermore, there was a significant increase in the number of CD16+ cells observed after co-incubation with nucleotide plus GM-CSF. In the presence of GM-CSF, the percentage of CD16+ cells in suspensions also containing ATP, Ap4A, or Ap3A were 37% (±4%), 47% (±7%), and 50% (±5%), respectively.

We then simultaneously measured both chromatin structure and CD16 expression in cultures incubated with GM-CSF and nucleotides. After 2 hours of incubation of control cells (no additions) over 90% of the cells exhibited high expression of CD16 and high propidium iodide fluorescence, indicating that <10% of the population showed signs of apoptosis (data not shown). However, by 24 hours incubation, >97% of the cells exhibited low CD16 expression and low propidium iodide fluorescence (Fig 6A). In suspensions containing GM-CSF, 23% of the cells had high CD16/propidium iodide staining (ie, were
Fig 4. Effect of adenosine deaminase on NADPH oxidase activity. (A) Neutrophils were incubated in the absence (control) or presence of adenosine deaminase (ADA, 1 U/mL), adenosine (50 μmol/L), or both together for 2 minutes before stimulation by 1 μmol/L fMet-Leu-Phe and measurement of luminol chemiluminescence. Values given are means (±SD, n = 3). (B) Neutrophils were incubated for 2 hours in the absence (control) or presence of adenosine deaminase (ADA, 1 U/mL), ATP (150 μmol/L), or GM-CSF (50 U/mL) or combinations thereof, as indicated. NADPH oxidase activity was then stimulated by 1 μmol/L fMet-Leu-Phe and measured by luminol chemiluminescence. Mean values are presented (±SD, n = 3).

Fig 5. Effects of GM-CSF, Ap4A, Ap3A, and ATP on neutrophil chemiluminescence. Neutrophils were incubated for 24 hours and NADPH oxidase was stimulated by the addition of 0.1 μg/mL PMA and measured by luminol chemiluminescence. Suspensions were incubated in the absence (control) or presence of GM-CSF with: (A) ATP, (B) Ap4A, and (C) Ap3A. All suspensions contained equal numbers of Trypan blue-excluding cells. (C) controls (no additions), (C) GM-CSF alone, (B) nucleotide alone, and (B) nucleotide plus GM-CSF. Typical traces from at least five separate experiments.

DISCUSSION

Regulation of neutrophil function by apoptosis has clear advantages for both the activation and resolution of the acute inflammatory response. Circulating cells constitutively undergo apoptosis and so are rapidly cleared from the circulation. However, in response to the generation of pro-inflammatory signals by endothelial cells or other immune cells, apoptosis is delayed so that the functional life span of neutrophils is extended. Upon resolution of the inflammatory re-
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Fig 6. Effects of GM-CSF and Ap2A, on CD16 expression and chromatin structure. Neutrophils were incubated for 24 hours in the absence (A) or presence of GM-CSF (B), Ap2A (C), or GM-CSF + Ap2A (D). After this incubation, chromatin structure was analyzed by propidium iodide staining in suspensions that were simultaneously stained for expression of CD16. Similar results were obtained in three other experiments.

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The diadenosine polyphosphates Ap3A and Ap4A and adenosine triphosphate interact with granulocyte-macrophage colony-stimulating factor to delay neutrophil apoptosis: implications for neutrophil: platelet interactions during inflammation

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