

Mechanisms Underlying Reduced Apoptosis in Neonatal Neutrophils

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ABSTRACT

Apoptosis, which leads to phagocytosis by mononuclear cells, represents the primary mechanism for removing neutrophils from inflamed tissues and minimizing injury. The present studies show that membrane phosphatidylserine turnover and permeability, as well as DNA fragmentation, were reduced in neutrophils from neonates when compared with adults. The activity of caspase 3 and expression of the proapoptotic proteins Bax, Bad, and Bak were also decreased in neonatal relative to adult neutrophils. These findings are consistent with impaired apoptosis in neonatal cells, which may contribute to prolonged inflammation in infants after oxidative stress or infection. Neutrophil apoptosis is induced by endogenous ligands such as Fas (FasL), which engage death receptors of the tumor necrosis factor/nerve growth factor

superfamily, including Fas receptor (FasR). We found that expression of FasR was decreased in neonatal when compared with adult cells. Moreover, neonatal neutrophils did not undergo apoptosis in response to anti-FasR antibody and exhibited impaired chemotaxis to soluble FasL. However, in both adult and neonatal cells, p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase inhibitors blocked Fas-induced activity. These data suggest that prolonged survival of neonatal neutrophils at injured sites is due, in part, to reduced responsiveness to FasL. This may be related to decreased expression of both FasR and Bcl-2-family proteins that mediate neutrophil apoptosis. (*Pediatr Res* 57: 56–62, 2005)

Human neutrophils are the primary effector cells in acute inflammation and are rapidly recruited from the bloodstream to injured sites. Although neutrophils from newborns exhibit defects in chemotaxis, phagocytosis, and oxidative metabolism (1–4), infants are at high risk for neutrophil-mediated tissue injury. Activated neutrophils are cleared from inflamed sites by the process of apoptosis, followed by macrophage phagocytosis. This promotes resolution rather than persistence of tissue injury (5,6). Attenuation of neutrophil apoptosis in neonates may contribute to severe and prolonged inflammatory responses. In premature infants, this may play a role in conditions such as bronchopulmonary dysplasia and necrotizing enterocolitis, which result in significant mortality and morbidity.

Neutrophil longevity and functional activity are regulated by inflammatory mediators present in the microenvironment.

Whereas proinflammatory cytokines, such as interferon- γ (IFN- γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), and bacterial-derived lipopolysaccharide (LPS), promote neutrophil activation and survival, anti-inflammatory mediators, such as IL-10 (IL-10) and Fas ligand (FasL), are proapoptotic (7–10). These mediators regulate the expression of pro- and antiapoptotic mitochondrial proteins, including Bak, Bad, Bax, and A1, as well as the proapoptotic effector protease caspase 3 (10–14). Previous studies have demonstrated that the rate of apoptosis is reduced in peripheral blood neutrophils from neonates relative to adult cells (15,16). We speculated that this is due to developmental alterations in the activity of inflammatory mediators and/or apoptotic signaling pathways, and this was investigated in the present studies.

METHODS

Reagents. LPS (serotype 0128:B12), *N*-formyl methionyl leucyl phenylalanine, and Hanks' balanced salt solution (HBSS) were obtained from Sigma Chemical Co. (St. Louis, MO). FITC-linked Annexin V was from R & D Systems (Minneapolis, MN), and propidium iodide and wortmannin were from Calbiochem (San Diego, CA). Rabbit polyclonal antibodies to Bax, Bak, Bad, and A1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Murine monoclonal anti-human Fas receptor (FasR), CD14, FasL, and purified mouse anti-human Fas IgM were from Upstate Biotechnology (Lake Placid, NY). FITC-labeled goat anti-mouse or sheep anti-rabbit IgG secondary anti-

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bodies were obtained from BD Transduction Laboratories (Lexington, KY) and New England Biolabs (Beverly, MA). Rabbit IgG and mouse IgG1 and IgM controls were purchased from Santa Cruz Biotechnology and Coulter Immunotech (Miami, FL). Human recombinant soluble FasL was from Alexis Corp. (Montreal, Quebec, Canada), and IFN- γ was from Invitrogen (Carlsbad, CA). LY 294002 and SB 203580 were from Biomol (Plymouth Meeting, PA).

Neutrophil isolation. These studies were approved by the Institutional Review Board of UMDNJ-Robert Wood Johnson Medical School. Blood (10 mL) was collected from umbilical cords immediately after delivery of healthy term (≥ 37 wk gestational age) infants. Adult peripheral blood samples were obtained from healthy volunteers. Neutrophils were isolated by dextran sedimentation, followed by Ficoll gradient centrifugation and hypotonic lysis of erythrocytes, as previously described (17).

Measurement of apoptosis. Neutrophils were washed in PBS, resuspended in Dulbecco's modified Eagle's medium (DMEM) that contained 10% fetal bovine serum, and then incubated in a shaking water bath at 37°C for 3–24 h in the absence or presence of LPS (100 ng/mL), anti-FasR antibody (20 ng/mL), IFN- γ (10 ng/mL), or GM-CSF (50 ng/mL). In some experiments, cells were preincubated with SB 203580 (33 μ M, 1 h), an inhibitor of p38 mitogen-activated protein (MAP) kinase, or with LY 294002 (50 μ M, 5 min), an inhibitor of phosphatidylinositol 3-kinase (PI3-K), before analysis of apoptosis. Three methods were used to assess neutrophil apoptosis: Annexin V binding, appearance of cytoplasmic histone-associated DNA fragments, and a transferase-mediated dUTP nick-end labeling (TUNEL) assay. For Annexin V binding studies, cells were centrifuged and resuspended (2×10^6 cells/mL) in buffer [10 mM of HEPES (pH 7.4), 140 mM of NaCl, and 2.5 mM of CaCl₂]. The cells were then incubated (15 min, room temperature) with Annexin V (1:20) and propidium iodide (1:10) and analyzed by flow cytometry on a Coulter EPICS Profile II (Hialeah, FL). Viable, apoptotic, and necrotic neutrophil populations were gated electronically, and data were analyzed using quadrant statistics based on relative Annexin V and propidium iodide fluorescence.

Cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) were quantified using a kit from Roche Applied Science (Indianapolis, IN). Neutrophils (1×10^5 cells/mL) were incubated in DMEM that contained 10% fetal bovine serum (37°C, 20 h), centrifuged, resuspended (5×10^4 cells/mL) in lysis buffer, and incubated at room temperature for 30 min. Cell lysates were centrifuged at $200 \times g$ for 10 min, and aliquots of the supernatants that contained cytoplasmic extracts were transferred to a streptavidin-coated microtiter plate (20 μ L/well). Samples were then incubated with biotin-linked anti-histone antibody and peroxidase-linked anti-DNA antibody (2 h, room temperature) and washed. After addition of the chromogen 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), histone-associated DNA was quantified by absorbance at 405 nm using a microplate reader.

TUNEL assays were performed using a kit from Roche Applied Science (Indianapolis, IN). Neutrophils were centrifuged and fixed by suspension in 4% paraformaldehyde (2×10^7 /mL; pH 7.4) for 1 h at room temperature. Cells were then washed in PBS, permeabilized by incubation with 0.1% Triton-X-100 in 0.1% sodium citrate for 2 min on ice, and incubated (37°C, 60 min) in a solution that contained terminal deoxynucleotidyl transferase (TdT) and fluorescein-labeled nucleotides. Negative controls were incubated in the absence of TdT, and positive controls were incubated in the presence of DNase I [3 U/mL in 50 mM of Tris-HCl (pH 7.5) and 1 mg/mL of BSA] for 10 min before labeling. Samples were then analyzed by flow cytometry.

Measurement of caspase 3 activity. Neutrophils (1×10^6 /mL) were incubated at 37°C for 3–24 h in DMEM that contained 10% fetal bovine serum. Cells were then treated (1 h, 37°C) with the fluorophore-linked peptide substrate GDEVDGI (PhiPhiLux-G₁D₂ kit; Oncolmmunin, Inc., Gaithersburg, MD), washed, and analyzed by flow cytometry. Cellular fluorescence is proportional to the amount of substrate cleaved by caspase 3 (18). Caspase 3 activity was also measured using a colorimetric assay (ApoAlert Caspase-3; Clontech, Palo Alto, CA). After incubation for 24 h, neutrophil lysates were microcentrifuged (3 min) and supernatants that contained soluble cellular proteins were incubated with substrate (DEVD) linked to the chromophore p-nitroanilide (37°C, 1 h). Samples were analyzed spectrophotometrically on a microplate reader at 405 nm.

Immunofluorescence. Neutrophils that were suspended in PBS that contained 1% BSA and 0.01% sodium azide (1.5×10^6 /mL) were incubated for 60 min at room temperature with a 1:1000 dilution of antibody to FasR, CD14,

FasL, or control (IgG1 or normal rabbit antisera); washed; and then incubated with FITC-labeled goat anti-mouse or rabbit IgG. After 30 min, the cells were analyzed by flow cytometry. For quantification of intracellular antigens, neutrophils were fixed with 0.1% paraformaldehyde; permeabilized with lysophosphatidylcholine (4 μ M, 30 min); and incubated overnight with a 1:500 dilution of anti-Bak, anti-Bax, anti-Bad, or anti-A1 antibody or IgG control. Cells were then washed, incubated with FITC-labeled goat anti-rabbit IgG (1:500, 30 min), and analyzed. Fluorescence histograms were analyzed by Overton's cumulative subtraction routine of the Coulter Cytologic Software program.

Measurement of neutrophil chemotaxis. Migration of neutrophils through Nucleopore polycarbonate filters was assayed using the modified Boyden chamber technique (19). For these studies, we used a 48-well microchemotaxis chamber (Neuro Probe, Inc., Pleasanton, CA). Thirty microliters of HBSS that contained 0.5% BSA, with or without soluble FasL (0.1 nM), was placed in each well of the lower chamber. A 5- μ m pore-size polycarbonate filter was then placed over the wells, and the upper chamber was set into place. Fifty microliters of neutrophils (1×10^5 cells) in HBSS that contained 0.5% BSA and 2.4 mg/mL of HEPES (pH 7.2) was added to each well. After incubation for 45 min at 37°C, the filter that contained adhered, migrated neutrophils was removed and stained with Wright-Giemsa. Chemotaxis was quantified as the number of cells that migrated through the filter in 10 oil immersion fields. In some experiments, cells were incubated at room temperature with SB 203580 (33 μ M, 1 h), LY 294002 (50 μ M, 5 min), or wortmannin (100 nM, 10 min) before analysis of chemotactic responsiveness. Data are presented as the mean \pm SE of four experiments. Mean background migration in response to HBSS was subtracted from each value before analysis.

Data analysis. Statistical analysis was performed using Statistica 5.5 (StatSoft, Inc., Tulsa, OK). The effects of treatments by group were compared by 2×4 ANOVA. Post hoc analysis was performed using the LSD planned comparison test. A $p < 0.05$ was considered statistically significant.

RESULTS

In initial studies, we compared spontaneous apoptosis in neonatal and adult neutrophils. One of the earliest signs of apoptosis is translocation of phosphatidylserine from the inner to the outer surface of the plasma membrane, which can be assessed by the binding of Annexin V (20). Significant Annexin V binding was detectable in neutrophils from both adults and neonates (Fig. 1). Binding increased with time in culture, reaching a maximum at 24 h. Apoptosis, as measured by

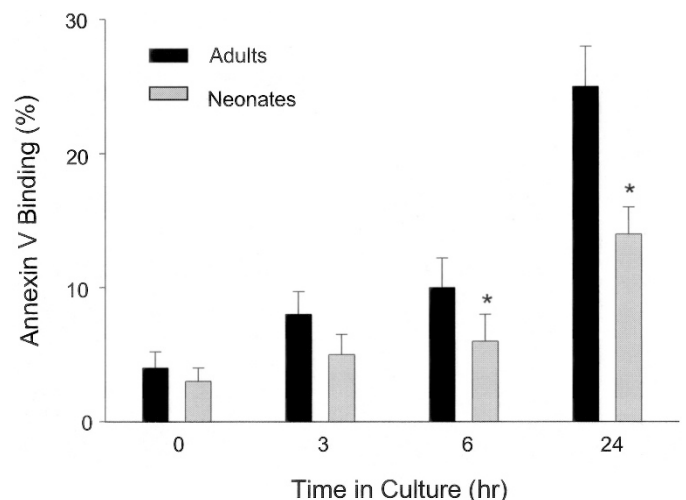


Figure 1. Spontaneous apoptosis in adult and neonatal neutrophils. Cells were cultured for 0–24 h before labeling with Annexin V and propidium iodide and analyzed by flow cytometry. The percentage of apoptotic cells was quantified, and data were analyzed using Coulter quadrant statistics based on relative fluorescence binding of Annexin V. Results are the mean \pm SE of four samples. *Significantly different ($p < 0.05$) from adults.

Annexin V binding, was significantly reduced in neonatal when compared with adult cells after 6 and 24 h in culture. In both neutrophil populations, viability decreased rapidly after 24 h in culture and by 48 h was <50%. Therefore, all subsequent analyses were performed on cells that were cultured for 24 h.

A biochemical hallmark of apoptosis is fragmentation of genomic DNA (21). This process is irreversible and occurs at later stages of apoptosis. Histone-associated DNA fragments were detectable in adult and neonatal neutrophils that were cultured for 24 h (Fig. 2, *top*). This activity was reduced ~60% in neonatal when compared with adult neutrophils. This was confirmed using a TUNEL assay, which detects DNA strand breaks by end-labeling DNA with fluorescein-dUTP. Flow

cytometric analysis of dUTP revealed two subpopulations of neutrophils that exhibited relatively low and high levels of DNA fragmentation (Fig. 2, *middle*). The percentage of cells that exhibited higher levels of DNA fragmentation was significantly reduced in neonatal neutrophils.

We next analyzed potential mechanisms that mediate delayed apoptosis in neonatal neutrophils. This has not been investigated previously. Cytoplasmic proteins of the Bcl-2 family regulate mitochondrial membrane permeability and the release of cytochrome *c* from the mitochondria into the cytoplasm. This is a rate-limiting step in early apoptosis (13). Whereas antiapoptotic Bcl-2 proteins such as A1 stabilize the mitochondrial membrane and inhibit the release of cytochrome *c*, proapoptotic proteins such as Bak, Bax, and Bad increase membrane permeability and promote its release (11,22). The relative expression of Bcl-2 proteins is thought to be a major determinant of apoptosis in neutrophils (23). We found that Bak, Bax, and Bad, as well as A1, were expressed in both adult and neonatal neutrophils. However, levels of the proapoptotic proteins were lower in cells from neonates (Fig. 3). In contrast, expression of the antiapoptotic protein A1 was similar in adult and neonatal neutrophils. During apoptosis, release of cytochrome *c* from the mitochondria triggers the cleavage of procaspase 9, which leads to activation of caspase 3, a key proapoptotic enzyme (24). Caspase 3 activity was detectable in both adult and neonatal neutrophils (Fig. 2, *bottom*); however, this activity was significantly reduced in neutrophils from neonates relative to adults.

Expression of Bcl-2 proteins in neutrophils is regulated by pro- and anti-inflammatory mediators that are present during the onset and resolution of tissue injury (23,25). We found that apoptosis was decreased by 35–55% in both adult and neonatal cells after treatment with the proinflammatory mediators IFN- γ and GM-CSF (Fig. 4). No significant differences were noted in the responses of the two cell populations to these mediators. Similarly, no differences were observed in the antiapoptotic effects of bacterially derived LPS on adult and neonatal neutrophils. This is consistent with the finding that expression of CD14, a glycosphosphatidyl-linked membrane-bound receptor for LPS, was similar in adult and neonatal cells (Fig. 5).

FasL is an endogenous proapoptotic protein generated by mononuclear cells and neutrophils (26). It initiates its biologic activity by binding to “death receptors” of the tumor necrosis factor/nerve growth factor receptor superfamily (27). Neutrophils from both adults and neonates were found to constitutively express membrane-bound FasL and its receptor, FasR (Fig. 5). Whereas expression of membrane-bound FasL was comparable in adult and neonatal neutrophils, levels of FasR were lower on neonatal cells. Treatment of neutrophils with anti-FasR antibody, which activates FasR (28), resulted in a 2-fold increase in apoptosis in adult cells (Fig. 6). In contrast, the antibody had no significant effect on apoptosis in neonatal cells. FasL was found to induce neutrophil chemotaxis in both adult and neonatal neutrophils (Fig. 7). This activity was significantly reduced in neonatal neutrophils relative to adult cells. Binding of FasL to FasR initiates a signaling cascade that leads to activation of p38 MAP kinase and PI3-K (29,30). Treatment of adult neutrophils with the p38 MAP kinase

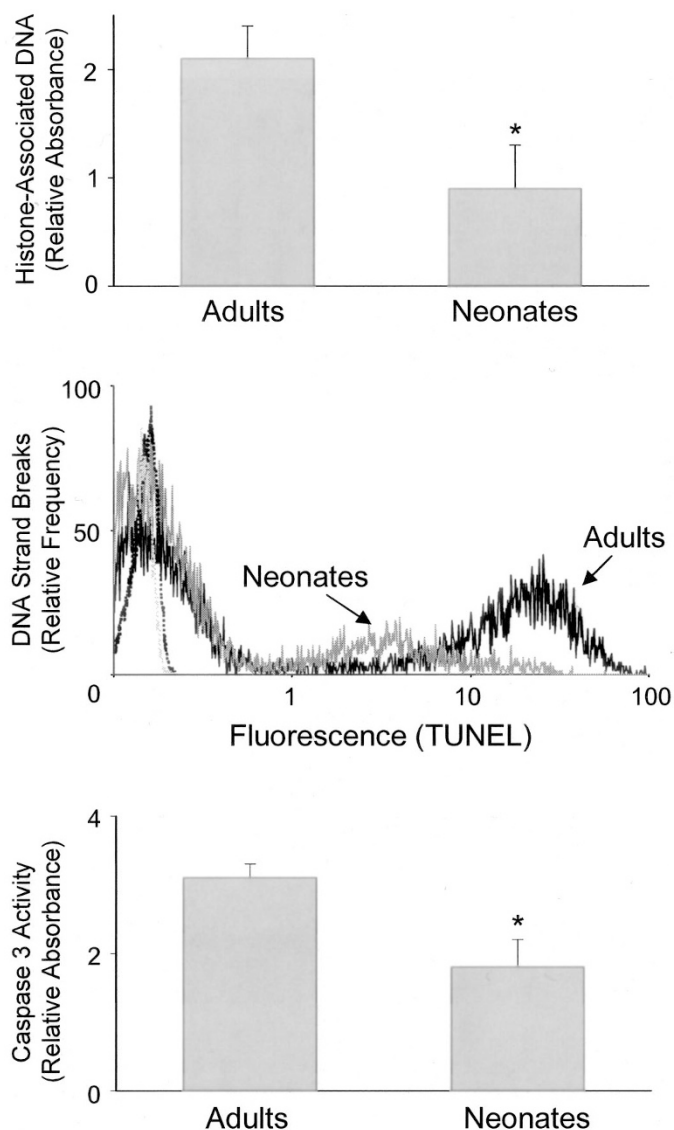


Figure 2. Comparison of apoptosis in adult and neonatal neutrophils. Isolated neutrophils were assayed for markers of apoptosis, as described in “Methods.” (*Top*) Histone-associated DNA fragments. (*Middle*) Fluorescence TUNEL assay. One representative sample from adults and neonates is shown. Negative controls were neutrophils that were incubated in the absence of TdT (dotted lines). (*Bottom*) Caspase 3 activity. The bars represent the mean \pm SE of four samples. *Significantly different ($p < 0.05$) from adults.

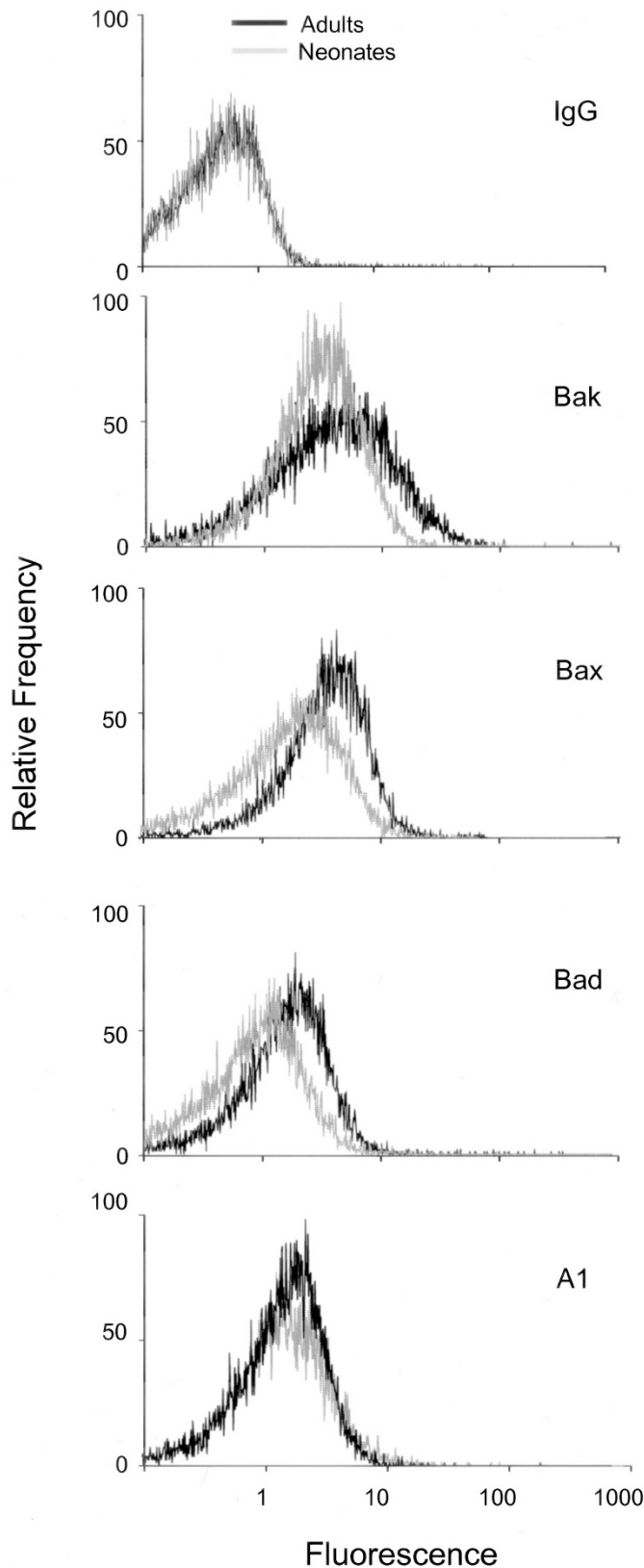


Figure 3. Comparison of pro- and antiapoptotic protein expression in adult and neonatal neutrophils. Neutrophils were analyzed by indirect immunofluorescence and flow cytometry using IgG control or primary antibodies against Bak, Bax, Bad, or A1 and FITC-labeled secondary antibody. One representative histogram for each antigen from three experiments in adult (black) and neonatal (gray) cells is shown.

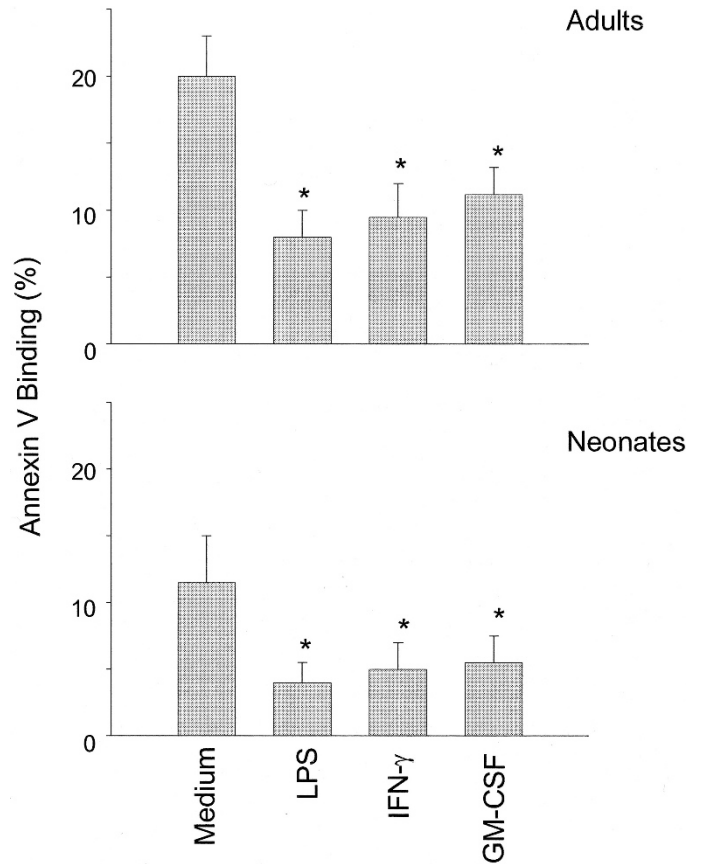


Figure 4. Effects of inflammatory mediators on neutrophil apoptosis. Neutrophils were incubated for 24 h in the presence of medium, LPS (100 ng/mL), IFN- γ (10 ng/mL), or GM-CSF (50 ng/mL). Cells were then labeled with Annexin V and propidium iodide and analyzed by flow cytometry. The percentage of apoptotic cells was quantified, and data were analyzed using Coulter quadrant statistics based on relative fluorescence binding of Annexin V. Each bar represents the mean \pm SE of four samples. *Significantly different ($p < 0.05$) from medium control.

inhibitor SB 203580 or the PI3-K inhibitor LY 294002 abrogated the proapoptotic effects of anti-FasR (Fig. 6). FasL-induced chemotaxis was also significantly reduced in adult cells by these inhibitors, as well as by wortmannin, which, like LY 294002, blocks PI3-K activity. Similar effects of the p38 MAP kinase and PI3-K inhibitors were observed on chemotaxis in neonatal cells (Fig. 7). The inhibitors alone had no effects on neutrophil apoptosis or chemotaxis (data not shown).

DISCUSSION

Inflammatory diseases in newborns are characterized by the persistence of neutrophils in injured tissues (5,6). This is thought to contribute to the pathogenesis of neonatal conditions such as bronchopulmonary dysplasia and necrotizing enterocolitis. In the present studies, we compared the longevity of neutrophils from neonates with adults and investigated potential mechanisms underlying differences in this activity. We found that several markers of apoptosis, including histone-associated DNA fragments and strand breaks, as well as caspase 3 activity, are reduced in neonatal neutrophils when compared with adults. These findings are novel and consistent

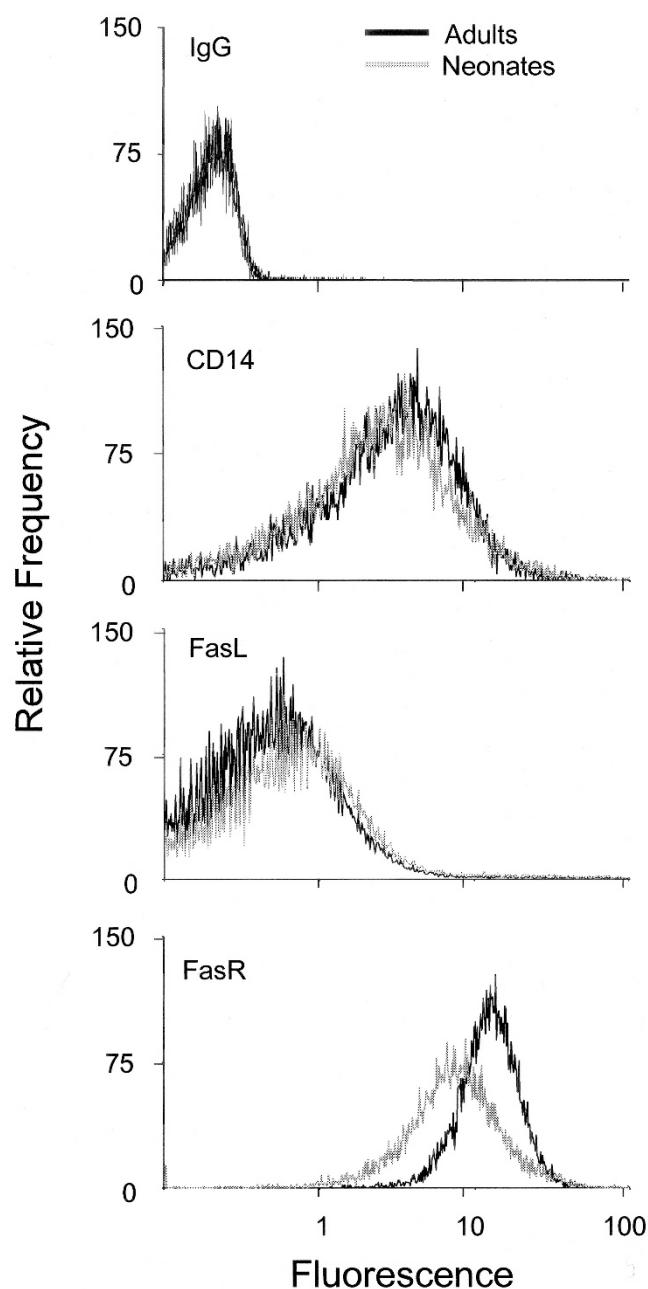


Figure 5. Expression of CD14, FasL, and FasR on adult and neonatal neutrophils. Cells were analyzed by indirect immunofluorescence and flow cytometry using primary antibodies against CD14, FasL, FasR, or IgG control and FITC-labeled secondary antibody. One representative histogram for each antigen from three experiments in adult (black) and neonatal (gray) cells is shown.

with previous studies demonstrating that apoptosis is delayed in neutrophils from neonates (16). Prolonged survival of neonatal neutrophils may play a role in pathologic inflammation in the lung, gastrointestinal tract, and other organs after exposure of infants to noxious stimuli.

Bcl-2-family proteins alter mitochondrial integrity and regulate the activity of caspases (23,25). Whereas proapoptotic Bcl-2 proteins act directly on the mitochondrial membrane resulting in the release of cytochrome *c*, antiapoptotic proteins such as A1 block the formation of apoptotic membrane pores,

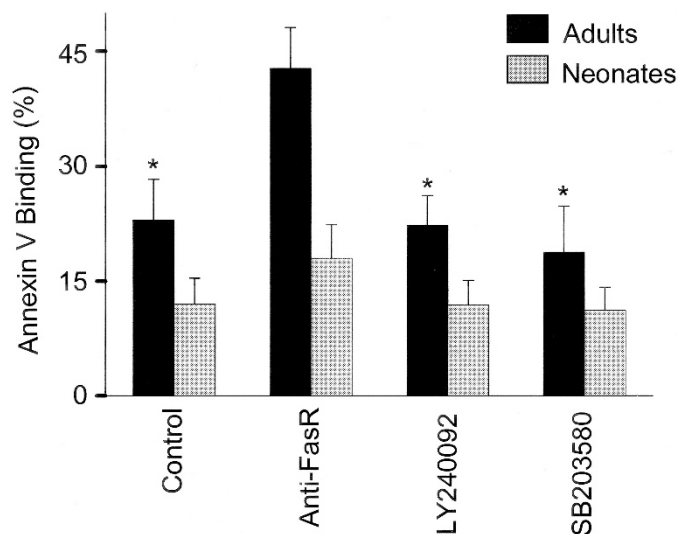


Figure 6. Fas-induced apoptosis in adult and neonatal neutrophils. Neutrophils were incubated for 24 h with medium control or with anti-FasR antibody (20 ng/mL) alone or in combination with LY 294002 (50 μ M) or SB 203580 (30 μ M). Cells were then labeled with Annexin V and propidium iodide and analyzed by flow cytometry. The percentage of apoptotic cells was quantified and data analyzed using Coulter quadrant statistics based on relative fluorescence binding of Annexin V. Each bar represents the mean \pm SE of four samples. *Significantly different ($p < 0.05$) from anti-FasR.

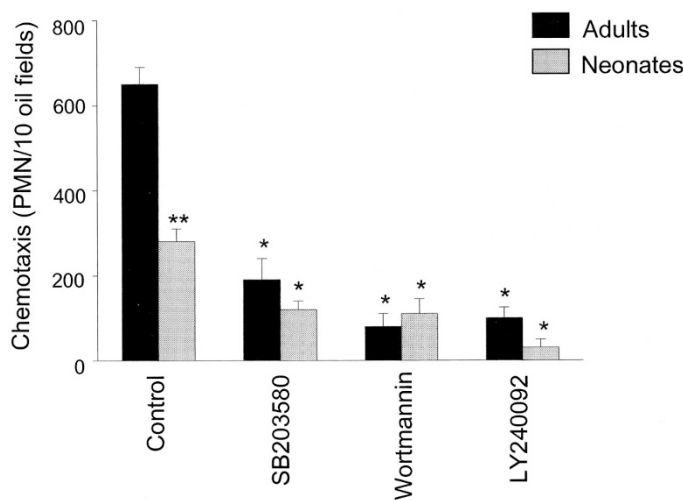


Figure 7. Induction of chemotaxis by soluble FasL. Cells were preincubated with medium control, SB 203580 (30 μ M, 1 h), LY 294002 (50 μ M, 5 min), or wortmannin (100 nM, 10 min) before assessment of chemotactic responsiveness to soluble FasL. In the absence of chemoattractant, random motility was 21.1 ± 5.2 for adult and 17.7 ± 4.8 for neonatal neutrophils. Each value represents the mean \pm SE of four samples. *Significantly different ($p < 0.05$) from control; **significantly different ($p < 0.05$) from adult cells.

effectively maintaining mitochondrial integrity (31). In adult neutrophils, expression of proapoptotic Bcl-2 proteins is relatively high compared with antiapoptotic proteins. This may account, in part, for the short lifespan and high turnover of neutrophils in the circulation (11). The present studies demonstrate that expression of the proapoptotic proteins Bax, Bak, and Bad was reduced in neonatal neutrophils relative to adult cells. In contrast, A1 expression was similar in both cell types. Reduced expression of proapoptotic Bcl-2-family proteins may underlie impaired apoptosis in neonatal neutrophils.

Inflammation resolves when proinflammatory signals diminish and proapoptotic anti-inflammatory signals are generated (26). FasL is a member of the tumor necrosis factor family that exhibits both pro- and anti-inflammatory activity (28,32). Whereas in the initial stages FasL promotes inflammation by stimulating neutrophil accumulation in tissues (33,34), during the resolution phase, binding of FasL to its receptor induces apoptosis (26,28). We found that Fas-induced chemotaxis and apoptosis were reduced in neonatal relative to adult neutrophils. This is associated with decreased expression of FasR on neonatal cells, which may account for deficits in the response of these cells to FasL. Our findings are in accordance with reports suggesting that impaired signaling *via* FasR can contribute to impaired resolution of the inflammatory response in newborns (35). Down-regulation of FasL, as well as caspases, has also been noted during chronic inflammation, which is characterized by persistent neutrophil activity (36). That FasL-induced chemotaxis was also impaired in neonatal cells suggests that alternative pathways that mediate neutrophil accumulation in tissues may be up-regulated.

Neutrophil apoptosis is regulated in part by mediators such as GM-CSF, IFN- γ , LPS, and FasL that are present during the early and resolution phases of the inflammatory response (37). Consistent with previous studies (7), we found that these proinflammatory mediators significantly reduced neutrophil apoptosis. This may contribute to the ability of neutrophils to remain viable at sites of injury or infection. Our findings that the decrease in apoptosis observed after treatment of the neutrophils with GM-CSF, IFN- γ , and LPS is similar in adult and neonatal cells suggest that these mediators play an essential role in regulating the inflammatory response in both cell types. GM-CSF, IFN- γ , and LPS signaling involves phosphorylation of PI3-K and p38 MAP kinase, leading to neutrophil activation (38). These pathways are also activated by binding of FasL to FasR and expression of FasR is up-regulated after activation of PI3-K (39–41). This suggests that PI3-K and p38 MAP kinase may play a role in FasR/FasL signaling in neutrophils. We found that blocking the activity of either PI3-K or p38 MAP kinase significantly decreased Fas-induced apoptosis and chemotaxis in adult cells. Fas-induced chemotaxis was also inhibited in neonatal cells, demonstrating that PI3-K and p38 MAP kinase are important in both responses in these cells. The observation that there were no differences between adult and neonatal cells in the effects of the inhibitors on chemotaxis suggests that alternative signaling pathways may mediate deficits in neonatal neutrophil functioning.

Our findings that expression of FasR, caspase 3, and proapoptotic Bcl-2-family proteins is reduced in neonatal neutrophils indicate that specific developmental deficiencies are responsible for delayed apoptosis in these cells. Delayed apoptosis in neonatal neutrophils may lead to prolonged inflammation and tissue injury, accounting, in part, for the severity of inflammatory diseases observed in newborns despite reduced neutrophil function. For example, neutrophil apoptosis followed by macrophage clearance is known to be important in the resolution of oxygen-induced lung injury, and lung neutrophils recovered from patients with acute respiratory distress syndrome exhibit markedly reduced apoptosis (15,42).

An understanding of the factors that regulate neutrophil apoptosis is essential for the development of more specific and effective therapeutic and preventive strategies in neonatal inflammatory diseases.

REFERENCES

- Hill HR 1987 Biochemical, structural, and functional abnormalities of polymorphonuclear leukocytes in the neonate. *Pediatr Res* 22:375–382
- Qing G, Rajaraman K, Bortolussi R 1995 Diminished priming of neonatal polymorphonuclear leukocytes by lipopolysaccharide is associated with reduced CD14 expression. *Infect Immun* 63:248–252
- Bortolussi R, Howlett S, Rajaraman K, Halperin S 1993 Deficient priming activity of newborn cord blood-derived polymorphonuclear neutrophilic granulocytes with lipopolysaccharide and tumor necrosis factor- α -triggered with formyl-methionyl-leucyl-phenylalanine. *Pediatr Res* 34:243–248
- Abughali N, DUBYAK G, Tosi MF 1993 Impairment of chemoattractant-stimulated hexose uptake in neonatal neutrophils. *Blood* 82:2182–2187
- Savill J 1997 Apoptosis in resolution of inflammation. *J Leukoc Biol* 61:375–380
- Savill J, Haslett C 1995 Granulocyte clearance by apoptosis in the resolution of inflammation. *Semin Cell Biol* 6:385–393
- Akgul C, Moulding DA, Edwards SW 2001 Molecular control of neutrophil apoptosis. *FEBS Lett* 487:318–322
- Renshaw SA, Timmons SJ, Eaton V, Usher LR, Akil M, Bingle CD, Whyte MK 2000 Inflammatory neutrophils retain susceptibility to apoptosis mediated via the Fas death receptor. *J Leukoc Biol* 67:662–668
- Tortorella C, Piazzolla G, Spaccavento F, Antonaci S 1998 Effects of granulocyte-macrophage colony-stimulating factor and cyclic AMP interaction on human neutrophil apoptosis. *Mediators Inflamm* 7:391–396
- Simon HU 2003 Neutrophil apoptosis pathways and their modifications in inflammation. *Immunol Rev* 193:101–110
- Moulding DA, Akgul C, Derouet M, White MR, Edwards SW 2001 BCL-2 family expression in human neutrophils during delayed and accelerated apoptosis. *J Leukoc Biol* 70:783–792
- Santos-Beneit AM, Mollinedo F 2000 Expression of genes involved in initiation, regulation, and execution of apoptosis in human neutrophils and during neutrophil differentiation of HL-60 cells. *J Leukoc Biol* 67:712–724
- Hengartner MO 2000 The biochemistry of apoptosis. *Nature* 407:770–776
- Hsieh SC, Huang MH, Tsai CY, Tsai YY, Tsai ST, Sun KH, Yu HS, Han SH, Yu CL 1997 The expression of genes modulating programmed cell death in normal human polymorphonuclear neutrophils. *Biochem Biophys Res Commun* 233:700–706
- Oei J, Lui K, Wang H, Henry R 2003 Decreased neutrophil apoptosis in tracheal fluids of preterm infants at risk of chronic lung disease. *Arch Dis Child Fetal Neonatal Ed* 88:F245–F249
- Allgaier B, Shi M, Luo D, Koenig JM 1998 Spontaneous and Fas-mediated apoptosis are diminished in umbilical cord blood neutrophils compared with adult neutrophils. *J Leukoc Biol* 64:331–336
- Ferrante A, Thong YH 1980 Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leukocytes from human blood by the Hypaque-Ficoll method. *J Immunol Methods* 36:109–117
- Watanabe M, Hitomi M, van der Wee K, Rothenberg F, Fisher SA, Zucker R, Svoboda KK, Goldsmith EC, Heiskanen KM, Nieminen AL 2002 The pros and cons of apoptosis assays for use in the study of cells, tissues, and organs. *Microsc Microanal* 8:375–391
- Boyden S 1962 The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. *J Exp Med* 115:453–466
- Sgonc R, Gruber J 1998 Apoptosis detection: an overview. *Exp Gerontol* 33:525–533
- Nagata S 2000 Apoptotic DNA fragmentation. *Exp Cell Res* 256:12–18
- Orlofsky A, Weiss LM, Kawachi N, Prystowsky MB 2002 Deficiency in the anti-apoptotic protein A1-alpha results in a diminished acute inflammatory response. *J Immunol* 168:1840–1846
- Weinmann P, Gaeltgens P, Walzog B 1999 Bcl-X1 and Bax- α -mediated regulation of apoptosis of human neutrophils via caspase-3. *Blood* 93:3106–3115
- McDonnell MA, Wang D, Khan SM, Vander Heiden MG, Kelekar A 2003 Caspase-9 is activated in a cytochrome c-independent manner early during TNF- α -induced apoptosis in murine cells. *Cell Death Differ* 10:1005–1015
- Dibbert B, Weber M, Nikolaizik WH, Vogt P, Schoni MH, Blaser K, Simon HU 1999 Cytokine-mediated Bax deficiency and consequent delayed neutrophil apoptosis: a general mechanism to accumulate effector cells in inflammation. *Proc Natl Acad Sci USA* 96:13330–13335
- Liles WC, Klebanoff SJ 1995 Regulation of apoptosis in neutrophils—Fas track to death? *J Immunol* 155:3289–3291
- Mannick JB, Hausladen A, Liu L, Hess DT, Zeng M, Miao QX, Kane LS, Gow AJ, Stamler JS 1999 Fas-induced caspase denitrosylation. *Science* 284:651–654
- Lile WC, Kiener PA, Ledbetter JA, Aruffo A, Klebanoff SJ 1996 Differential expression of Fas (CD95) and Fas ligand on normal human phagocytes: implications for the regulation of apoptosis in neutrophils. *J Exp Med* 184:429–440
- Alvarado-Kristensson M, Porn-Ares MI, Grethe S, Smith D, Zheng L, Andersson T 2001 p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase activities have opposite effects on human neutrophil apoptosis. *FASEB J* 16:129–131
- Webb PR, Wang KQ, Scheel-Toellner D, Pongracz J, Salmon M, Lord JM 2000 Regulation of neutrophil apoptosis: a role for protein kinase C and phosphatidylinositol-3-kinase. *Apoptosis* 5:451–458

31. Tsujimoto Y 2003 Cell death regulation by the Bcl-2 protein family in the mitochondria. *J Cell Physiol* 195:158–167
32. Brown SB, Savill J 1999 Phagocytosis triggers macrophage release of Fas ligand and induces apoptosis of bystander leukocytes. *J Immunol* 162:480–485
33. Ottonello L, Tortolina G, Amelotti M, Dallegri F 1999 Soluble Fas ligand is chemotactic for human neutrophilic polymorphonuclear leukocytes. *J Immunol* 162:3601–3606
34. Seino K, Iwabuchi K, Kayagaki N, Miyata R, Nagaoka I, Matsuzawa A, Fukao K, Yagita H, Okumura K 1998 Chemotactic activity of soluble Fas ligand against phagocytes. *J Immunol* 161:4484–4488
35. Leuenroth S, Lee C, Grutkoski P, Keeping H, Simms HH 1998 Interleukin-8-induced suppression of polymorphonuclear leukocyte apoptosis is mediated by suppressing CD95 (Fas/Apo-1)-Fas-1 interactions. *Surgery* 124:409–417
36. Le'Negrate G, Rostagno P, Auberger P, Rossi B, Hofman P 2003 Downregulation of caspases and Fas ligand expression, and increased lifespan of neutrophils after transmigration across intestinal epithelium. *Cell Death Differ* 10:153–162
37. Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A 1992 Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* 80:2012–2020
38. Burg ND, Pillinger MH 2001 The neutrophil: function and regulation in innate and humoral immunity. *Clin Immunol* 99:7–17
39. O'Brien D, O'Connor T, Shanahan F, O'Connell J 2002 Activation of the p38 MAPK and ERK1/2 pathways is required for Fas-induced IL-8 production in colonic epithelial cells. *Ann NY Acad Sci* 973:161–165
40. Coulter KR, Doseff A, Sweeney P, Wang Y, Marsh CB, Wewers MD, Knoell DL 2002 Opposing effect by cytokines on Fas-mediated apoptosis in A549 lung epithelial cells. *Am J Respir Cell Mol Biol* 26:58–66
41. Ivanov VN, Krasilnikov M, Ronai Z 2002 Regulation of Fas expression by STAT3 and c-Jun is mediated by phosphatidylinositol 3-kinase-AKT signaling. *J Biol Chem* 277:4932–4944
42. Matute-Bello G, Liles WC, Radella F 2nd, Steinberg KP, Ruzinski JT, Jonas M, Chi EY, Hudson LD, Martin TR 1997 Neutrophil apoptosis in the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 156:1969–1977