Immunity, Vol. 5, 653–666, December, 1996, Copyright ©1996 by Cell Press

A Novel Role for the β2 Integrin CD11b/CD18 in Neutrophil Apoptosis: A Homeostatic Mechanism in Inflammation

Angela Coxon,* Phillipe Rieu,‡§ Fern J. Barkalow,*§ Sanaz Askari,† Arlene H. Sharpe,* Ulrich H. von Andrian,† M. Amin Arnaout,‡ and Tanya N. Mayadas*
*Department of Pathology
Brigham and Women's Hospital
†Center for Blood Research
and Department of Pathology
Harvard Medical School
‡Leukocyte Biology and Inflammation Program
Renal Division
Massachusetts General Hospital
Harvard Medical School
Boston, Massachusetts 02115

Summary

In mice selectively deficient in CD11b/CD18, a β2 integrin, chemoattractant-induced leukocyte adhesion to microvascular endothelium in vivo was reduced. Paradoxically, thioglycollate-induced neutrophil accumulation in the peritoneal cavity was increased and was associated with a significant delay in apoptosis of extravasated cells. The extravasated cells had a near absence of neutrophil phagocytosis and a reduction in oxygen free radical generation, which may contribute to the observed defect in apoptosis. This is supported by our in vitro studies, in which phagocytosis of opsonized particles by human neutrophils rapidly induced apoptosis that could be blocked with CD11b/CD18 antibodies. Reactive oxygen species are the intracellular link in this process: phagocytosis-induced apoptosis was blocked both in neutrophils treated with the flavoprotein inhibitor diphenylene iodonium and in neutrophils from patients with chronic granulomatous disease, which lack NADPH oxidase. Thus, CD11b/CD18 plays a novel and unsuspected homeostatic role in inflammation by accelerating the programmed elimination of extravasated neutrophils.

Introduction

Neutrophils are a prominent component of the acute inflammatory response. The control of neutrophil efflux, mediated in part by endothelial and leukocyte adhesion receptors, is important in regulating inflammation and tissue damage. Neutrophil diapedesis occurs following leukocyte rolling on the vessel wall promoted by selectins, and firm adhesion of rolling leukocytes is mediated by members of the β2 integrin family (Carlos and Harlan, 1994). β2 integrins are four heterodimers with distinct α subunits (CD11a through CD11d) sharing a β subunit (CD18, β2). These receptors are dramatically up-regulated on the surface of activated leukocytes and mediate various leukocyte adhesion–dependent events. Deficiency of β2 integrins (leukocyte adhesion deficiency type I), caused by mutations in the common CD18 subunit, predisposes to life-threatening infections, owing to impaired intravascular adhesion, transendothelial migration, phagocytosis, and target cell killing (Anderson and Springer, 1987; Arnaout, 1990a). Since all β2 integrins are deficient in these patients, the relative biologic importance of each of these heterodimers in the various leukocyte adhesion defects was derived indirectly utilizing subunit-specific function-blocking monoclonal antibodies (MAbs). These studies suggest that CD11b/CD18 (Mac-1, CR3, Mo1, $\alpha_m\beta2$) expressed by polymorphonuclear cells, macrophages, and natural killer cells is the most versatile of the β2 integrins. Among its many functions, CD11b/CD18 mediates firm adhesion of activated neutrophils to endothelium and epithelium through interaction with intercellular adhesion molecules (ICAMs) on the endothelium and other, as yet unidentified, endothelial and epithelial ligands. In vivo modulation of these adhesion receptors has been effective in reducing leukocyte efflux and limiting the inflammatory response (Carlos and Harlan, 1994).

Equally important to limiting the inflammatory response are factors involved in its resolution. One component of resolution is the clearance of neutrophils and their potentially histotoxic contents. The major pathway of neutrophil clearance is programmed cell death followed by engulfment by macrophages. Large numbers of apoptotic neutrophils and neutrophil-ingesting macrophages have been observed in experimental peritonitis induced by thioglycollate broth (Sanui et al., 1982) and other models allowing frequent sampling of the cellular infiltrate (Chapes and Haskill, 1983; Cox et al., 1995). In humans, apoptotic neutrophils, both within macrophages as well as free in the inflammatory milieu, have been identified in inflammatory diseases (Firestein et al., 1995; Grigg et al., 1991).

Several cytokines and chemotactic and priming agents present during inflammation prolong neutrophil survival (Colotta et al., 1992; Lee et al., 1993; Liles and Klebanoff, 1995). However, the rapid turnover of neutrophils in inflammed tissue suggests the existence of a mechanism(s) that triggers apoptosis in these cells. Elucidation of cell surface molecules that regulate apoptosis in extravasated neutrophils is still in its infancy. Although apoptosis in peripheral blood neutrophils (PBN) can be induced by antibody ligation of Fas (Iwai et al., 1994; Liles et al., 1996; Liles and Klebanoff, 1995), a death-promoting gene in lymphocytes (Abbas, 1996), the importance of this molecule in programmed cell death of extravasated neutrophils in not known. The transgenic expression of the anti-apoptotic protein bcl-2 in neutrophils inhibits apoptosis in thioglycollate-elicited neutrophils in vitro (Lagasse and Weissman, 1994). However, neutrophils do not normally express bcl-2 (Afford et al., 1992; Iwai et al., 1994). Several activation-dependent neutrophil functions lead to changes in second messenger systems that are associated with induction of apoptosis in neutrophils. These include transient elevations of cytosolic free calcium, oxygen radical generation, and protein tyrosine phosphorylation (Gottlieb

§ These authors contributed equally to this work.

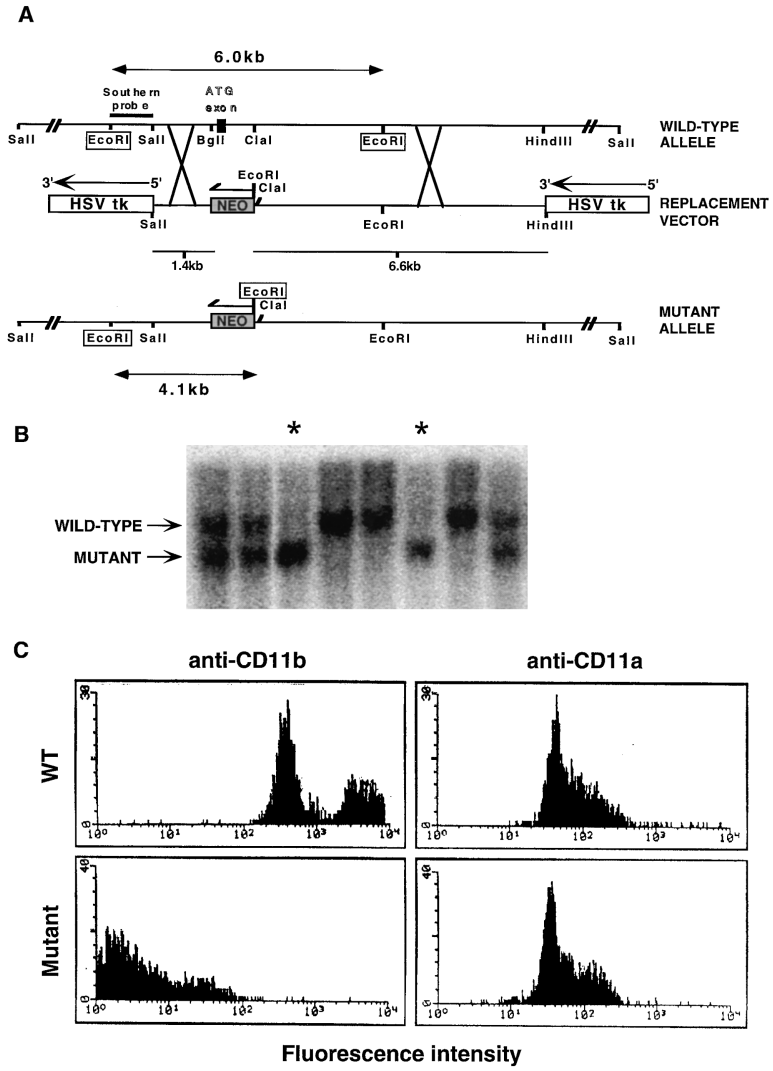

Figure 1. Targeted Disruption of the CD11b Gene Locus

(A) The CD11b locus (wild-type allele) and the replacement vector are shown. To construct the replacement vector, a BglI–ClaI (800 bp) fragment, which includes the 64 bp ATG exon, was deleted and replaced with a 1.7 kb neomycin gene cassette driven by a PGK promoter. The 5′ flanking probe used for screening embryonic stem cell clones and genotyping mice is indicated (Southern probe). The probe detects a 6.0 kb EcoRI fragment from the wild-type allele and a 4.1 kb fragment from the mutant allele.

(B) Southern blot analysis of EcoRI-digested tail DNA from a litter of a heterozygous intercross. Fragments obtained from wild-type and mutant alleles are indicated. Two mice are homozygous for the mutation (asterisk).

(C) Thioglycollate-elicited leukocytes from wild-type (WT) and CD11b/CD18-deficient mice (Mutant) were stained with Gr-1 MAb to identify granulocytes and anti–murine CD11b or CD11a MAb. CD11b and CD11a expression was assessed on the Gr-1-positive population. This is a representative experiment of three performed. CD11b was not detectable in mutant neutrophils, but CD11a was similar to wild type.

et al., 1995; Watson et al., 1996; Whyte et al., 1993; Yousefi et al., 1994). Consequently, it is likely that genes that can influence other aspects of neutrophil behavior are also involved in active cell death. In this regard, there is growing evidence that engagement of CD11b/CD18 promotes phagocytosis, spreading, chemotaxis, and transduction of intracellular signals that lead to changes in calcium flux, gene expression, and the oxidative burst (Arnaout, 1990b).

In this study we examined the biological roles of CD11b/CD18 in neutrophil functions in vivo by generating mice deficient in expression of this receptor. Analyses of these mice revealed several neutrophil defects consistent with previous known functions of this adhesion receptor, as well as a novel, unanticipated role in apoptosis of extravasated neutrophils.

Results

Generation of CD11b/CD18-Deficient Mice and Verification of CD11b/CD18 Deficiency

A targeting vector was constructed in which the exon encoding the translational initiation codon (ATG) and 15 amino acids of the signal peptide were deleted from the CD11b genomic clone and replaced with the neomycin resistance positive selection marker (Figure 1A). Mice heterozygous for the mutation were generated using standard protocols. Homozygous animals resulting from heterozygous matings were born at approximately 20.4% frequency, which is close to the expected Mendelian frequency (25%). Genotypes of a litter resulting from one of these crosses are shown (Figure 1B). Homozygous animals appear grossly normal, and their viability up to 18 months of age suggests that CD11b/CD18 is not essential for embryonic development or leukogenesis. We also observed no increase in infections in the CD11b/CD18-deficient colony under viral-free conditions but otherwise not quarantined, suggesting that these mice are not severely immunocompromised.

Neutrophils and resident macrophages were assessed for surface CD11b/CD18 expression and other granulocyte markers. Extravasated mutant peritoneal neutrophils had no detectable CD11b/CD18 expression but wild-type levels of the sister integrin CD11a/CD18 (Figure 1C). In data not shown, peripheral blood leukocytes from mutant mice were negative for CD11b/CD18

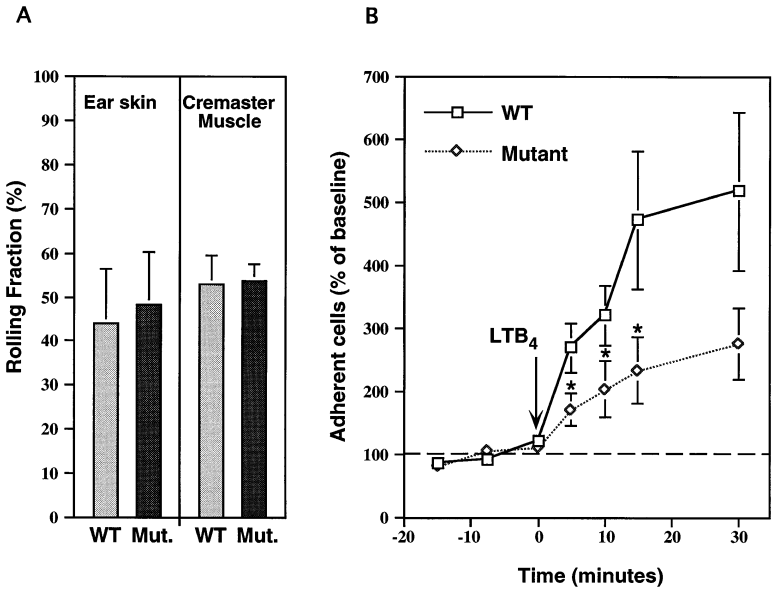

Figure 2. Intravital Leukocyte Rolling and Firm Adhesion

(A) Leukocyte rolling in venules of ear skin and cremaster muscle of wild-type (WT) and CD11b/CD18-deficient (Mutant) mice was studied. The rolling leukocyte fraction (i.e., the percentage of endogenous rolling cells over total leukocyte flux) was the same in wild type and mutant in both tissue preparations. Mean ± SEM from 18 (wild-type) to 36 (mutant) venules from three wild-type and mutant animals is shown.

(B) Leukocyte adhesion in the microcirculation of the cremaster muscle before and following LTB_4 superfusion was determined. Leukocytes that remained stationary in a venule for at least 30 s were defined as firmly adherent. The number of firmly adherent leukocytes per μm^2 at distinct timepoints before and during LTB_4 superfusion was expressed relative to background spontaneous adherence. This accounted for the heterogeneity in baseline sticking that was encountered in different venules of both wild-type and mutant animals. Background adherence was determined by averaging the number of adherent cells per 100 μm^2 in each venule during the 15 min control period and was set as 100% (broken line). Leukocyte adherence in the venules of cremaster muscle of the mutant mice (19 venules in three animals) was significantly reduced (asterisk, $p < 0.05$) compared with wild-type (18 venules in three animals).

but had wild-type levels of L-selectin. In addition, CD11b/CD18 expression was absent on F4/80-positive peritoneal macrophages harvested from mutant mice 5 days after intraperitoneal injection of thioglycollate (data not shown).

CD11b/CD18 Deficiency Does Not Affect Circulating Leukocyte, Neutrophil, and Platelet Counts under Basal Conditions

Blood leukocyte counts in mutants were comparable with wild type (wild type, 6310 ± 550 leukocytes per microliter [n = 33]; mutant, 5490 ± 370 leukocytes per microliter [n = 32]), as were PBN counts (wild type, 1180 ± 150; mutant, 1020 ± 140). This suggests that CD11b/CD18 deficiency does not contribute to the leukocytosis observed either in patients with defects in all β2 integrins (Anderson and Springer, 1987; Arnaout, 1990a) or in mice with reduced levels of β2 integrin expression (Wilson et al., 1993). The number of platelets was similar in mutants and wild type (wild type, 9.2 ± 0.4 × 10^8 platelets per milliliter [n = 16]; mutant, 9.9 ± 0.5 × 10^8 platelets per milliliter [n = 16]), which indicates that platelet homeostasis is not perturbed in mutant mice.

CD11b/CD18 Deficiency Does Not Affect Neutrophil Rolling, but Markedly Inhibits Leukotriene B_4-Induced Firm Adhesion in the Microcirculation

Leukocyte rolling and firm adhesion were examined in two intravital microscopy preparations. Loss of CD11b/CD18 had no effect on the ability of neutrophils to roll on endothelial cells in the ear or cremaster muscle microcirculations (Figure 2A). The velocity of rolling cells was comparable between wild-type and null mice (data not shown). In contrast, the ability of null neutrophils to adhere firmly to the endothelium in the cremaster muscle in response to leukotriene B_4 was significantly impaired (Figure 2B).

Increased Neutrophil Accumulation in CD11b/CD18 Null Mice in Thioglycollate-Induced Peritonitis Is Associated with Decreased Apoptosis of Extravasated Neutrophils

To determine the role of CD11b/CD18 in leukocyte accumulation in response to an experimental model of peritonitis, CD11b/CD18-deficient and wild-type animals were given an intraperitoneal injection of thioglycollate, an effective inducer of a neutrophil-rich inflammatory exudate (Lewinsohn et al., 1987). As early as 2 hr, mutant animals had 2-fold more neutrophils in the peritoneal exudate compared with wild-type mice, and by 10.5 hr mutants had 3-fold higher neutrophil levels than wild type (Figure 3A). The increase in neutrophil accumulation may be due to a decrease in clearance of these cells.

The predominant pathway of neutrophil clearance following thioglycollate-induced peritonitis is apoptosis followed by phagocytosis of the apoptotic neutrophils (Sanui et al., 1982). The phagocytosis of apoptotic neutrophils by mutant and wild-type peritoneal macrophages was indistinguishable (A. C. and T. N. M., unpublished data). We studied whether neutrophil apoptosis was defective in extravasated mutant neutrophils. Neutrophil apoptosis is characterized by specific morphological changes such as nuclear condensation and fragmented nuclei, which are the gold standard for identifying apoptotic neutrophils (Savill et al., 1989). It is also characterized by a reduction in DNA content detected by propidium iodide staining of fixed, permeabilized cells (a hypodiploid DNA peak present upon flow cytometric analysis), the appearance of a DNA "ladder," and a decrease in cell surface expression of CD16 (FcγRIII) (Dransfield et al., 1994). We assessed the percentage of

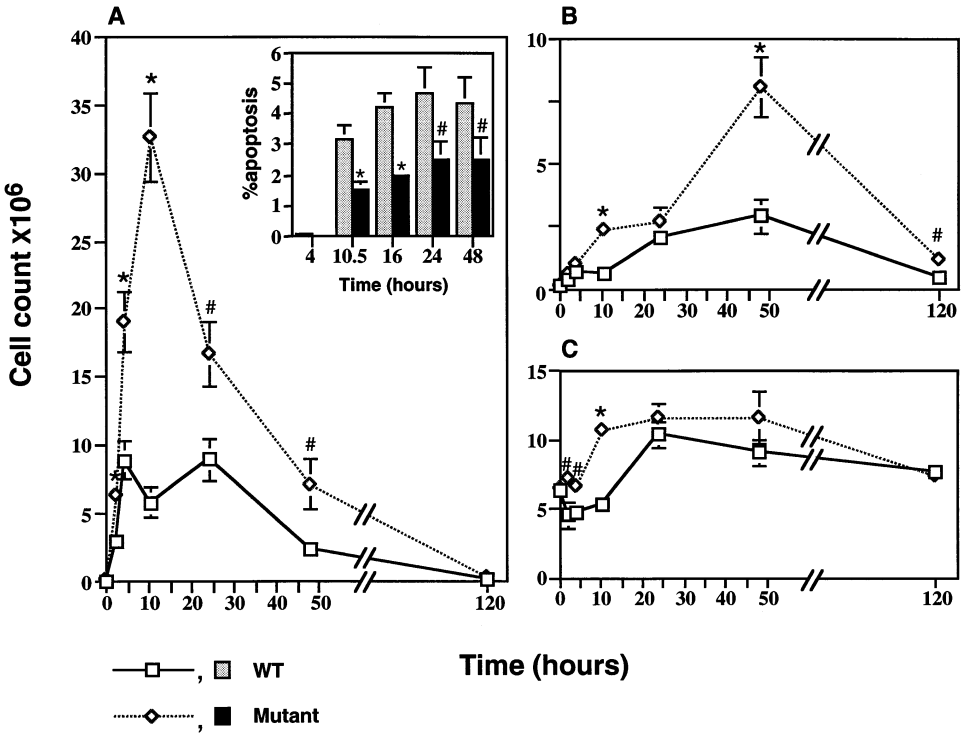

Figure 3. Peritoneal Leukocyte Accumulation and Percentage of Apoptotic Neutrophils after Thioglycollate Administration

At the indicated times after intraperitoneal injection of thioglycollate, cells were harvested from the peritoneal cavity, and the number of neutrophils (A), eosinophils (B), and mononuclear cells (C) in the lavage was determined. Inset in (A), stained cytospin preparations of the peritoneal exudate were scored for percentage of apoptotic neutrophils using morphologic criteria. Significantly higher number of neutrophils accumulated in the peritoneum of mutant mice compared with wild type. The percentage of extravasated mutant neutrophils that were apoptotic was lower than wild type. Peritoneal eosinophils and mononuclear cells were also elevated in thioglycollate-treated CD11b/CD18 null mice compared with wild type. $n \geq 10$ wild-type and 10 mutant animals at each timepoint. Asterisk, $p < 0.005$; number sign, $p < 0.05$.

apoptotic neutrophils in cytospins of peritoneal lavage samples harvested from wild-type and mutant mice by morphological criteria. The percentage of apoptotic neutrophils in mutant lavage samples was lower than wild-type at several timepoints following thioglycollate treatment (Figure 3A, inset). The difference in neutrophil apoptosis between wild-type and mutant 10.5 hr lavage samples at the 0 hr timepoint was confirmed by propidium iodide staining and subsequent flow cytometric analysis of neutrophils purified from lavage samples (wild type, $9.7 \pm 0.15\%$; mutant, $2.2 \pm 0.5\%$ [$p < 0.0001$]).

Eosinophil accumulation in mutant animals was also higher than wild type at 10.5 hr, 48 hr, and 5 days after thioglycollate injection (Figure 3B). Increased mononuclear counts in the lavage of mutant mice were evident at the early timepoints (Figure 3C). The enhanced recruitment in mononuclear cells and eosinophils may be due to the increased accumulation of neutrophils. In fact, previous studies in a model of pulmonary inflammation suggest that the initial neutrophil accumulation is responsible for subsequent enhanced neutrophil recruitment and mononuclear cell accumulation (Henson, 1991). Indeed, we found that eosinophil and mononuclear cell peritoneal accumulation 10.5 hr following thioglycollate treatment was similar in mutant and wild-type mice rendered neutropenic with cyclophosphamide (wild type mononuclear, $3.48 \pm 0.5 \times 10^6$; eosinophils, $0.21 \pm 0.04 \times 10^6$; mutant mononuclear, $3.17 \pm 0.4 \times 10^6$; eosinophils, $0.19 \pm 0.04 \times 10^6$).

Apoptosis of Extravasated Peritoneal Neutrophils In Vitro Is Delayed in CD11b/CD18 Mutants

The percentage of apoptotic cells observed in freshly harvested peritoneal exudates of Figure 3A (inset) is low because apoptotic neutrophils are rapidly cleared by phagocytes in vivo. Therefore, we followed the apoptosis of thioglycollate-elicited neutrophils in vitro. We cultured the total leukocyte population present in the peritoneal lavage of animals treated for 2 hr with thioglycollate. These cells would presumably have received the "death-inducing" signal in vivo. The cells were then cultured under conditions that prevented adhesion of leukocytes to the tissue culture ware. At several timepoints, a morphological assessment of apoptosis on cytospins of samples was undertaken and cell viability was assessed. CD11b/CD18 null neutrophils had a 56% and 45% reduction in apoptosis in comparison with wild-type neutrophils after 6 and 8 hr in culture, respectively (Figure 4A).

In contrast with necrotic cells, cells undergoing apoptosis maintain integrity of their plasma membrane, particularly at early stages of programmed cell death, and therefore exclude trypan blue. Trypan blue exclusion was used to assess the percentage of viable cells, which include apoptotic cells and is a population distinct from necrotic cells, which are dye permeable (Savill et al., 1989). Cell viability remained at >90% during the experimental time period for both genotypes (Figure 4A).

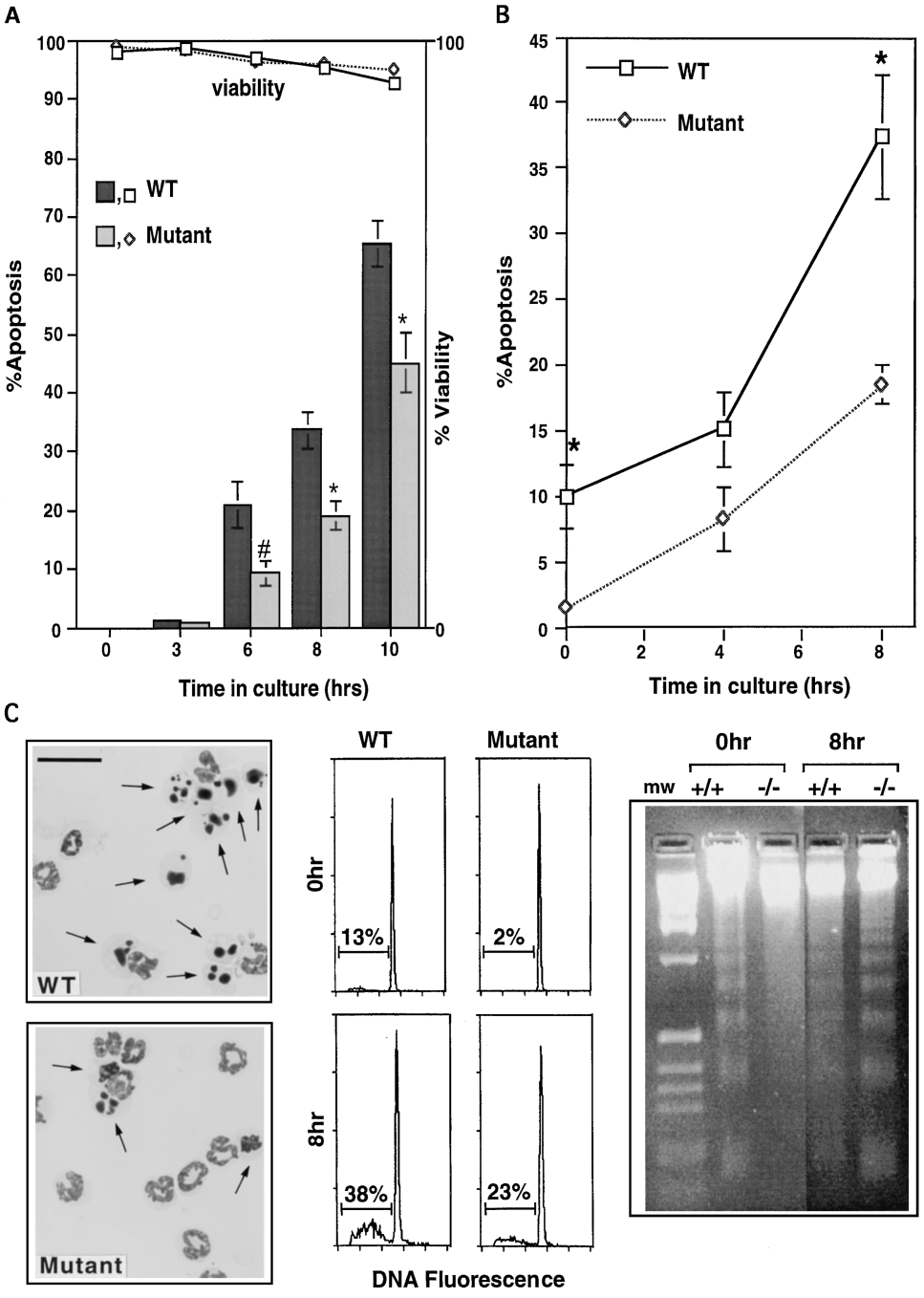

Figure 4. In Vitro Apoptosis of Extravasated Neutrophils

(A) At 2 hr after thioglycollate administration, peritoneal leukocytes from individual mice were cultured directly and assessed for apoptosis by morphological criteria. The results shown are the average of 11 wild-type and 12 mutant mice. CD11b/CD18-deficient neutrophils had a significant reduction in apoptosis after 6 and 8 hr in culture. Cell viability assessed by trypan blue exclusion was greater than 90% in both genotypes.

(B) At 10.5 hr after thioglycollate administration, peritoneal lavage was collected and neutrophils were isolated by density centrifugation. At time 0, neutrophils were placed in culture, and at the indicated timepoints apoptotic cells were scored on cytospins. The percentage of apoptotic neutrophils in the mutant sample was consistently lower than wild type. n = 8 experiments.

(C) Decreased apoptosis in mutants compared with wild-type was confirmed by three different methods of analyzing apoptosis. Representative experiments, included in (B), are shown. Left, stained cytospins from the 8 hr timepoint. Apoptotic neutrophils are indicated by arrows. Bar is 20 μm. Middle, FACS analysis of propidium iodide–stained cells. The percentage of stained cells present in the hypodiploid DNA peak was gated in each profile. DNA electrophoresis of the same samples is shown on the right. DNA laddering was present in wild-type samples (+/+) but absent in the null (−/−) samples at 0 hr. Laddering appeared in the null samples after 8 hr in culture. mw, 1 kb molecular weight marker. Asterisk, $p < 0.005$; number sign, $p < 0.05$.

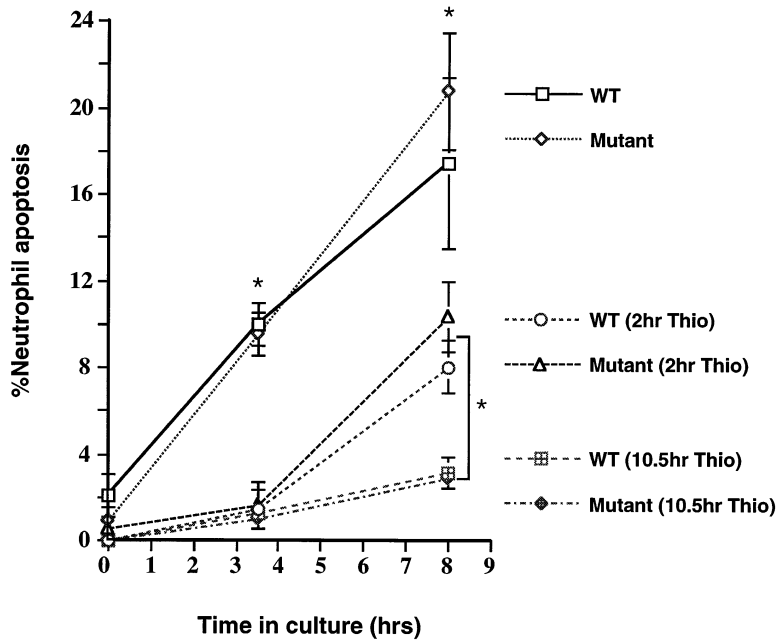

Figure 5. Spontaneous Apoptosis of PBN

PBN were isolated from blood of untreated wild-type and mutant mice and mice treated with thioglycollate for 2 and 10.5 hr as indicated. Neutrophils (>97% pure) were placed in culture. At the indicated timepoints, cells were collected and the percentage apoptosis was determined on cytospins. Each timepoint represents the average of 3 experiments. Asterisk, $p < 0.005$ between naive and thioglycollate-treated samples and between 2 hr and 10.5 hr thioglycollate-treated samples at the 8 hr timepoint.

This suggests that cellular processes that lead to loss in membrane integrity, i.e., cell necrosis, are not affected by CD11b/CD18 deficiency.

The differences in apoptosis between wild-type and mutant extravasated neutrophils were reproduced in neutrophils purified by density centrifugation from the peritoneal exudate of wild-type and mutant mice treated for 10.5 hr with thioglycollate. We observed a significant reduction in apoptotic neutrophils at the 0 hr timepoint, which is consistent with Figure 3A (inset), as well as after 8 hr in culture (Figure 4B). Apoptosis in the same samples was also documented using propidium iodide and DNA laddering. We observed a hypodiploid DNA peak in samples of null neutrophils stained with propidium iodide that was consistently smaller than wild type at both the 0 and 8 hr timepoints. Apoptosis was also qualitatively confirmed in these samples by DNA laddering (Figure 4C). The percentage of viable cells decreased to approximately 60% after 8 hr in culture, possibly as a result of the neutrophil isolation procedure, but was comparable between cultures of both genotypes.

Apoptosis of CD11b/CD18 Mutant PBN In Vitro Is Equivalent to Wild Type

To determine whether the decrease in apoptosis of extravasated neutrophils was a consequence of a younger population of circulating neutrophils in mutant mice, apoptosis in PBN isolated from wild-type and mutant animals was compared. In PBN harvested from untreated mice, the percentage of apoptosis in mutant neutrophils in culture was similar to wild type (Figure 5). This indicates that spontaneous apoptosis of blood neutrophils occurs via a CD11b/CD18-independent mechanism. In PBN collected from animals treated with thioglycollate for 2 hr and 10.5 hr, apoptosis in wild-type and mutant PBN was also similar (Figure 5). However, apoptosis in PBN isolated from thioglycollate-treated mice was significantly delayed compared with PBN isolated from naive mice. Cytokines and other inflammatory mediators have been previously shown in vitro to delay apoptosis of neutrophils (Squier et al., 1995). Thioglycollate-stimulated inflammatory mediators in blood may similarly inhibit apoptosis of PBN. It is also possible that thioglycollate-treated mice have increased release of the bone marrow pool of neutrophils into the circulation with the younger neutrophils, contributing to the observed delay in apoptosis.

Phagocytosis and Oxidative Burst Are Impaired in Thioglycollate-Elicited Neutrophils from CD11b/CD18 Mutants

An important function of extravasated neutrophils is the phagocytosis and killing of microorganisms. CD11b/CD18 has been previously shown to mediate phagocytosis of serum-opsonized particles, cell spreading, and the associated vigorous generation of the oxidative burst (Arnaout, 1990b). In the next several experiments, we tested our hypothesis that neutrophil apoptosis is induced through CD11b/CD18-mediated phagocytosis and its associated oxidative burst. This would represent a mechanism by which foreign pathogens are eliminated without risking serious tissue damage by the myriad of toxic chemicals used by neutrophils to destroy microorganisms.

We assessed the ability of CD11b/CD18 null neutrophils to phagocytose complement-opsonized particles, spread, and generate an oxidative burst. Thioglycollate-elicited CD11b/CD18-deficient neutrophils were unable to bind to iC3b, the major opsonin of the complement system (data not shown) and were unable to ingest complement-opsonized Oil-red-O particles. PBN spreading on glass (a form of "frustrated" phagocytosis) was also dramatically attenuated in CD11b/CD18 null neutrophils. In addition, the oxidative burst displayed by activated

Table 1. Effect of CD11b Deficiency on Neutrophil Phagocytosis, Spreading, and Oxidative Burst

	Wild Type	CD11b Null	Percent Reduction
Phagocytosis[a] (μg of Oil-Red-O/PMN/min)	20.00 ± 1.54	−1.37 ± 0.55*	100 (n = 3)
Spreading[b] (Percent cells)	86.31 ± 2.44	10.77 ± 2.40*	88 (n = 7)
Oxidative burst[c] (Mean fluorescence)	52.68 ± 12.51	20.85 ± 3.30#	60 (n = 6)

Phagocytosis and oxidative burst were assessed in thioglycollate-elicited neutrophils. Spreading was assessed on purified peripheral blood neutrophils.

[a] Phagocytosis. Cells were incubated with murine serum-opsonized Oil-Red-O for 7 min ± NEM, an inhibitor of phagocytosis. Values represent the rate of phagocytosis in samples incubated with opsonized Oil-Red-O in the absence of NEM minus samples incubated in the presence of NEM. Each of three individual experiments represents cells pooled from the lavage of six wild-type and six mutant thioglycollate-treated mice.

[b] Spreading of cells was assessed on a glass hemocytometer. Each sample represents purified PBN from one mouse.

[c] Oxidative burst activity was examined in cells incubated with Gr-1 MAb and DCFA. Oxidation of the nonfluorescent intracellular DCFA to fluorescent DCF in Gr-1 positive neutrophils was assessed by cytofluorimetric analysis. Values are the mean DCF fluorescence. Each sample contains cells harvested from the peritoneum of one 2 hr thioglycollate-treated mouse.

Values are the mean ± SEM. Asterisk, $p < 0.005$; number sign, $p < 0.05$.

neutrophils collected from the peritoneal lavage of thioglycollate-treated mice was reduced by >60% in mutant neutrophils (Table 1).

Phagocytosis of Serum-Opsonized Particles Induces Apoptosis via a CD11b/CD18-Dependent Mechanism

We next asked whether phagocytosis of complement-opsonized particles triggers neutrophil apoptosis, and whether the process is mediated by CD11b/CD18. Purified human PBN were incubated with or without serum-opsonized Oil-red-O particles. Cells were then washed, placed in culture, and assayed for neutrophil apoptosis at 0 and 4 hr using three different analytical methods of apoptosis: morphological features of apoptosis on cytospins, CD16 surface expression, and propidium iodide staining. Cell viability, assessed by trypan blue exclusion, was greater than 90% for all samples. By all the above criteria for apoptosis, neutrophils fed serum-opsonized particles had significantly higher levels of apoptosis compared with unfed neutrophils 4 hr after they were placed in culture (Figure 6A).

To test whether CD11b/CD18-mediated phagocytosis leads to apoptosis, purified human PBN were allowed to ingest serum-opsonized Oil-red-O particles in the presence or absence of a MAb directed against CD11b (MAb 44), which blocks phagocytosis by 70%–80% (Arnaout et al., 1983). A MAb against CD11a (Mentzer et al., 1986) served as a control. Samples were taken at 0, 2, 3, and 4 hr after culture. Apoptosis was assessed in duplicate slides of cells stained with Wright–Giemsa or acridine orange, the latter permitting the simultaneous assessment of ingestion as well as apoptosis in the same cell. As shown in Figure 6B, phagocytosis induced a dramatic increase in neutrophil apoptosis compared with cells incubated without opsonized particles. This was observed within 2 hr in culture. Microscopic examination of acridine orange–stained cells confirmed that apoptosis was present only in particle-containing cells (data not shown). The anti-CD11b MAb reduced phagocytosis-induced apoptosis by ~71% and ~76% at 2 and 3 hr, respectively (Figure 6B). The antibody had no effect on spontaneous apoptosis of neutrophils not fed opsonized particles (data not shown). Identical results were obtained with another phagocytosis blocking anti-CD11b MAb (MAb 107; Rieu et al., 1994), which is isotype matched with the anti-CD11a MAb (data not shown). Cell viability in samples was greater than 92% under all conditions. The rapid induction of neutrophil apoptosis upon CD11b/CD18-mediated phagocytosis is strong evidence that the early accumulation of neutrophils in the peritoneal cavity of mutant mice (see Figure 3) could be entirely due to a defect in neutrophil apoptosis and subsequent clearance.

NADPH Oxidase–Generated Oxygen Radicals Are Required for CD11b/CD18-Mediated Phagocytosis-Induced Apoptosis

Reactive oxygen intermediates (ROI) are implicated in apoptosis of several different cell types (Buttke and Sandstrom, 1994). In neutrophils, a striking increase in oxygen radical generation accompanies CD11b/CD18-mediated phagocytosis. This relies on the activity of NADPH oxidase, a multicomponent enzyme that catalyzes the transfer of electrons from reduced nicotinamide dinucleotide phosphate (NADPH) to molecular oxygen (Babior, 1992). To examine whether ROI are required for CD11b-mediated apoptosis, the effect of the flavoprotein inhibitor diphenylene iodonium (DPI) (Cross, 1990) was investigated. DPI effectively abrogated phagocytosis-induced superoxide generation (detected by NBT reduction; data not shown) without affecting phagocytosis (Figure 7A, inset). Strikingly, phagocytosis did not trigger apoptosis in these cells (Figure 7A). DPI also inhibits nitric oxide synthetase (Stuehr et al., 1991). Therefore, we also evaluated the role of ROI in PBN from patients with chronic granulomatous disease, who have a genetic deficiency in the NADPH oxidase (Dinauer et al., 1990). PBN from two unrelated patients (Dinauer et al., 1990; Peter Newburger, personal communication) did not undergo the respiratory burst as expected (data not shown). In both patients, phagocytosis did not induce apoptosis up to 5 hr after culturing compared with controls (Figure 7B).

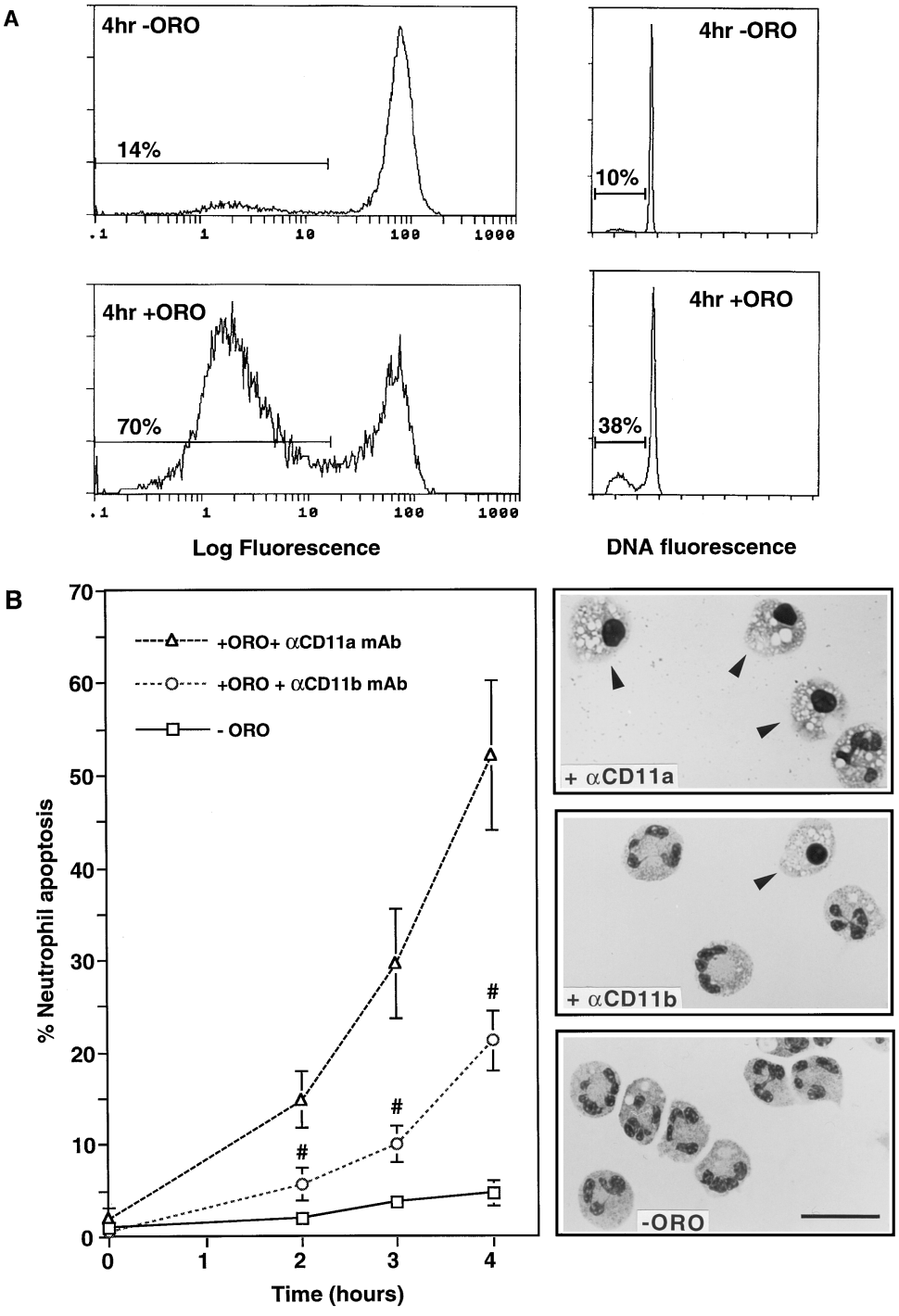

Figure 6. Human PBN Apoptosis after Phagocytosis

(A) Isolated human PBN were fed complement-opsonized Oil-red-O (plus ORO) particles or diluent alone (minus ORO). Cells were cultured (time 0 hr), and at 0 and 4 hr samples were processed for CD16 expression (left) and propidium iodide staining (right). Representative experiments are shown. Left, the percent of CD16-negative cells is shown. The results of four experiments indicate that at 0 hr there was no difference in the percentage of cells with reduced CD16 expression (minus ORO, 21 ± 6%; plus ORO, 28 ± 12%). At 4 hr, there was a significant increase in the number of CD16-negative cells in cell samples that had phagocytosed ORO (plus ORO) compared with unfed cells (minus ORO) (minus ORO, 19 ± 3%; plus ORO, 78 ± 6%), $p > 0.002$. Right, propidium iodide staining after 4 hr in culture shows that the percentage of hypodiploid nuclei is higher in cells fed ORO compared with those not fed ORO.

(B) Cells were preincubated with buffer, CD11a MAb, or CD11b MAb and then subjected to the complement-opsonized ORO phagocytosis assay described in (A); since the results of samples incubated with ORO alone were identical to those in which CD11a MAb was added prior to incubation with ORO, only the data for the latter are shown. Minus ORO samples were incubated without Oil-red-O or antibodies. At the indicated timepoints after plating, the percentage of cell apoptosis was assessed on cytospins. $n = 4$; asterisk, $p < 0.05$. Representative Wright–Giemsa-stained cytospin preparations of cells after 4 hr in culture are shown. Apoptotic cells are indicated by arrowheads. Phagocytic

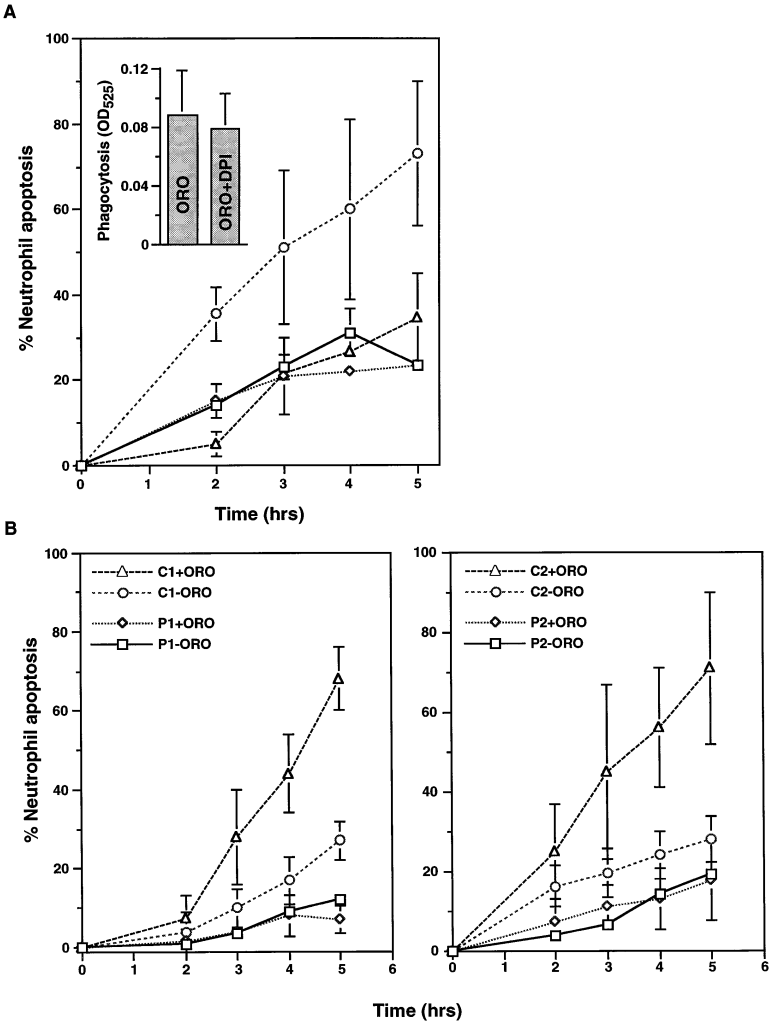

Figure 7. Phagocytosis-Induced Apoptosis in Neutrophils from Patients with CGD and in Normal Neutrophils Treated with a NADPH Oxidase Inhibitor

(A) Effect of DPI on ingestion and apoptosis. Human PBN were incubated at 37°C in buffer alone (diamonds) or buffer containing serum-opsonized ORO (circles) for 5 min in the absence (triangles) or presence (squares) of DPI (50 μM). Washed cells were then placed in culture, and aliquots were obtained at the indicated timepoints and assessed for apoptosis using morphologic criteria. Values are the average of three independent experiments. The effect of DPI on phagocytosis was determined simultaneously in parallel incubations (inset). Histograms represent values obtained after subtracting the background (phagocytosis in the presence of 1 mM NEM as described in Experimental Procedures).

(B) Effects of congenital absence of NADPH oxidase activity on CD11b-mediated apoptosis. Human PBNs from two patients with CGD (P1 and P2) and two controls (C1 and C2) were left in buffer or fed serum-opsonized ORO for 5 min at 37°C. Cells were then washed, cultured for the indicated times, and assessed for apoptosis as described in (A). Values are the average of two independent experiments.

This indicates that NADPH oxidase–derived ROI, triggered by CD11b/CD18-mediated phagocytosis, are responsible for neutrophil apoptosis.

Discussion

Our studies clearly demonstrate an important role for CD11b/CD18 in neutrophil functions such as chemoattractant-induced adhesion to the endothelium, phagocytosis, spreading (frustrated phagocytosis), and generation of an oxidative burst. More importantly, our studies reveal an unanticipated role for this receptor in neutrophil apoptosis as well as an underlying mechanism.

Mice generated with a mutation in the CD11b locus have no detectable levels of CD11b/CD18 on their neutrophils or macrophages. An absence of CD11b/CD18 does not affect expression levels of CD11a/CD18 or other neutrophil markers such as L-selectin and Gr-1. Deficiency of CD11b/CD18 has interesting features that distinguish it from deficiency of β2 integrins. CD11b/CD18 null mice have normal peripheral leukocyte counts and can extravasate into tissues such as the inflammed peritoneum. In contrast, the inflammed tissues of β2 integrin (CD18)-deficient humans (Anderson et al., 1985; Arnaout et al., 1982), dogs (Giger et al., 1987), and cattle (Kehrli et al., 1990) are largely devoid of neutrophils, despite a characteristic peripheral granulocytosis. Neutrophils of CD11b/CD18-deficient mice share phenotypes associated with β2-deficient neutrophils, thus delineating the functions that are primarily CD11b/CD18 dependent. Neutrophils from both groups display impaired spreading, complement iC3b binding, phagocytosis, and the associated oxidative burst. In vivo, we demonstrate that CD11b/CD18-deficient mice have a defect in neutrophil adhesion in the acute response to the neutrophil activating stimulus, leukotriene B_4. These findings are consistent with previous data showing that β2 integrins play little role in neutrophil rolling (von Andrian et al., 1993) and emphasize the role of CD11b/CD18 in neutrophil firm adhesion in response to neutro-

vacuoles and apoptosis are prominent in samples that have phagocytosed opsonized Oil-red-O particles (middle), but are largely absent in samples preincubated with MAb to CD11b prior to incubation with particles as well as in those not fed particles (left). Bar is 20 μm.

phil-activating stimuli (Arnaout et al., 1988; Mulligan et al., 1994). In previous studies using function-blocking MAbs, CD11b/CD18 was shown to promote leukocyte rolling at low shear rates (Gaboury and Kubes, 1994). Our studies do not support these findings: constitutive leukocyte rolling was unaffected in the ear in the absence of surgical trauma despite low shear rates present in this organ.

The apparently normal extravasation of CD11b/CD18 null neutrophils in the thioglycollate peritonitis inflammation model may reflect the overlapping roles of β2 integrins (Schmits et al., 1996), as well as the role of selectins (Arbones et al., 1994; Frenette et al., 1996; Mayadas et al., 1993) in neutrophil emigration in this model. This finding suggests that the reduction in intravascular adhesion observed by intravital microscopy in mutant mice is not a rate-limiting step in the complex sequence of events leading to intraperitoneal leukocyte accumulation. The increase we observed in the number of neutrophils accumulating in the peritoneum of thioglycollate-treated CD11b/CD18 null mice compared with the wild-type suggests that CD11b/CD18 may play a role in neutrophil survival. The number of apoptotic neutrophils present in the peritoneal cavity of mutant mice was significantly reduced compared with wild type, a difference that was reproduced in purified peritoneal neutrophils aged in culture. These data suggest an unanticipated role for CD11b/CD18 in promoting neutrophil apoptosis. The uptake of apoptotic neutrophils by peritoneal macrophages deficient in CD11b/CD18 is unaffected (A. C. and T. N. M., unpublished data), which is consistent with previous studies suggesting a role for the vitronectin receptor and phosphatidylserine receptor on macrophages in this process (Fadok et al., 1992; Savill et al., 1990).

A potential mechanism for the CD11b/CD18-dependent apoptosis in extravasated neutrophils is suggested by our in vitro studies, in which phagocytosis of complement-opsonized particles by normal human neutrophils induced apoptosis and blocking with anti-CD11b MAbs attenuated the effect. A similar delay in neutrophil apoptosis is undoubtedly present in β2 integrin–deficient cells, but is probably concealed by the concomitant defect in extravasation. Watson et al. (1996) reported that ingestion of serum-opsonized Escherichia coli by neutrophils leads to neutrophil apoptosis, although no role for CD11b/CD18 was demonstrated. Our studies indicate that this process is dependent on CD11b/CD18. The link of CD11b/CD18-dependent phagocytosis with apoptosis provides a novel counterregulatory mechanism to accelerate apoptosis in the inflammed tissue under external conditions that nurture neutrophil survival. Such a mechanism would eliminate phagocytic cells that have reached the end of their useful life span in inflammed tissues, thus limiting detrimental effects from toxic contents of neutrophils. Our contention that an absence of CD11b/CD18-mediated phagocytosis is the mechanism for the delay in apoptosis in the extravasated mutant neutrophils is supported by our findings that phagocytosis of serum-opsonized particles and neutrophil spreading (frustrated phagocytosis) are absent in mutant neutrophils. However, the particulate material engulfed by infiltrating neutrophils in thioglycollate-induced peritonitis has not been characterized. It is not essential that the particulate material is a CD11b/CD18 ligand, since previous studies suggest that CD11b/CD18 is required for phagocytosis even when there is no known CD11b/CD18 ligand present (Gresham et al., 1991). This may be related to the requirement for other phagocytic receptor–ligand complexes to interact with CD11b/CD18 to trigger phagocytosis and signal transduction events (Fallman et al., 1993; Petty and Todd, 1993; Zhou and Brown, 1994). Since CD11b/CD18-deficient extravasated neutrophils do eventually undergo apoptosis and are cleared in CD11b/CD18 null mice, it is probable that mechanisms other than CD11b/CD18-dependent phagocytosis can also trigger apoptosis.

CD11b-mediated particle ingestion and spreading induce or facilitate, respectively, ROI (Arnaout, 1990b; Nathan et al., 1989). Previous studies have shown that ROI can lead to cell death in several cell types (Busciglio and Yankner, 1995; Hockenbery et al., 1993; Um et al., 1996; Watson et al., 1996). Mutant neutrophils directly harvested from the peritoneal cavity have reduced oxygen radical content, which may be a consequence of their reduced phagocytic and spreading activities. The reduction in oxygen radical generation in mutant extravasated neutrophils may be a reason why they have a decrease in apoptosis. In fact, our in vitro studies provide strong evidence that oxygen radical generation associated with CD11b/CD18-mediated phagocytosis triggers apoptosis in neutrophils. Apoptosis following phagocytosis is severely compromised in neutrophils from patients with chronic granulomatous disease (CGD), which are unable to generate superoxide and related ROI since they lack a functional NADPH oxidase complex. This is despite a normal phagocytosis of opsonized particles by CGD neutrophils. The defect in apoptosis could also be reproduced in normal neutrophils in the presence of the NADPH oxidase inhibitor, DPI. The identification of a defect in apoptosis in neutrophils of CGD patients has implications for the clinical manifestations of CGD. It raises the possibility that the prolonged inflammatory response observed in patients with CGD may not be primarily due to a hyperactive immune response, infection, or the failure to degrade chemoattractants as previously suggested (Henderson and Klebanoff, 1983), but may be the result of prolonged neutrophil survival at sites of inflammation. Of interest, thioglycollate-induced peritonitis in mice deficient in specific components of the NADPH oxidase complex (Jackson et al., 1995; Pollock et al., 1995) led to a profile of increased neutrophil and leukocyte accumulation that is strikingly similar to our observations in the CD11b/CD18 null mice. The overall increase in leukocyte accumulation in these studies may be due to the initial neutrophil accumulation leading to a positive feedback for further leukocyte recruitment. This is supported by our findings in which we show that the exaggerated mononuclear and eosinophil accumulation in CD11b/CD18 null mice is eliminated if they are rendered neutropenic. Based on our studies, it is also possible that leukocyte subsets, some of which also express CD11b/CD18, have a delay in apoptosis owing to a lack of CD11b/CD18-mediated phagocytic activity and the associated oxidative burst.

The finding that CD11b/CD18-mediated phagocytosis induces apoptosis, whereas apoptosis of resting peripheral neutrophils occurs in the absence of CD11b/CD18, defines at least two apoptotic pathways for the disposal of neutrophils. These two pathways may be referred to as "activation-induced" and "spontaneous" apoptosis. CD11b/CD18 induces cell death in neutrophils that have been activated, thus playing an important regulatory role in down-regulating the acute inflammatory response and aiding in its resolution. This scenario has a striking similarity to one occurring in lymphocytes: Fas, a member of the tumor necrosis factor receptor family, induces apoptosis in activated T lymphocytes, playing a major role in down-regulating many immune functions, with a defective Fas system leading to autoimmunity (Abbas, 1996). Alterations in cell survival contribute to the pathogenesis of a number of human diseases from cancer to autoimmune disease (Thompson, 1995). Whether CD11b/CD18 deficiency predisposes to autoimmunity in certain conditions remains to be examined, but two recent findings suggest that a deficiency in this β2 integrin may lead to autoimmune disease: a patient with a mutation in the CD11b epitope responsible for binding complement-opsonized particles has systemic lupus erythematosus (Witte et al., 1993) and certain strains of β2 integrin–deficient mice have been recently reported to develop autoimmune skin disease (Bullard et al., 1996).

In conclusion, we demonstrate that CD11b/CD18 plays an important role in multiple neutrophil functions including an unanticipated function in apoptosis. CD11b/CD18-mediated apoptosis would ensure an orderly destruction of extravasated leukocytes, thus contributing to the resolution of the inflammatory lesion. Elucidation of the mechanisms that mediate leukocyte influx, as well as the mechanisms governing the normal resolution of inflammation, may lead to a greater understanding of the pathogenesis of acute and chronic inflammation since both are intrinsic components of many disease processes.

Experimental Procedures

Construction of CD11b Targeting Vector and Generation of CD11b-Deficient Mice

The mouse CD11b locus was cloned from a genomic library using a murine CD11b cDNA provided by Dr. Robert Pytela (University of California, San Francisco) (Pytela, 1988). The targeting vector was constructed by replacing a 800 bp BglI–ClaI fragment spanning the exon containing the ATG translational start codon with a 1.7 kb PGK–neo–poly(A) cassette. Herpes simplex virus thymidine kinase (HSV–tk) cassettes were ligated to the linear ends. The construct was linearized with NotI and used to electroporate 10^7 J1 ES cells (Li et al., 1992), which were maintained on a feeder layer of neo^r embryonic fibroblasts in the presence of 500 U/ml LIF. Clones were selected with G418 (400 μg/ml) and FIAU (2 μM) and were evaluated by Southern blot analysis of DNA with EcoRI and hybridization to external probes. Of 196 doubly resistant clones screened, 4 had undergone homologous recombination. Further analysis of these four clones with a number of restriction enzymes provided no evidence for other untoward genetic events. Three of the four clones were injected into 3.5 day C57Bl/6 or Balb/c blastocysts and produced chimeric mice. Agouti offspring of chimeric animals were analyzed for the presence of the mutated CD11b allele. Tail biopsies were used to prepare DNA by standard methods for Southern blot and PCR analysis (Laird et al., 1991). Animals were bred and maintained in a virus antibody–free animal facility at the Longwood Medical Research Center of the Harvard Medical School.

We analyzed elicited neutrophils for CD11a and CD11b expression 10.5 hr following thioglycollate injection. Leukocytes in the lavage were incubated with phycoerythrin-labeled Gr-1 (Pharmingen) to identify granulocytes and mouse CD11a or CD11b FITC-labeled rat MAbs (Pharmingen). Cells were subjected to two-color flow cytometric analysis on a FACScan flow cytometer (Becton Dickinson); 5000 cells were analyzed.

Analysis of Leukocyte Behavior by Intravital Microscopy

Animal Preparation

Adult mice were anesthetized with 0.25 ml of saline containing 12.5 mg/ml ketamine HCl (Ketaset, Fort Dodge Laboratories), 2.5 mg/ml xylazine (Rompun, Miles Inc.), and the right jugular vein (for rhodamine 6G injection) and left carotid artery (anesthesia injection as needed) were catheterized using heparinized polyethylene tubing (Intramedic PE-10, Becton Dickinson). Ear skin (ES): animals were placed in a supine position on a plexiglass stage and one ear positioned on a microscope slide was covered with glycerol and a glass coverslip (No. 1, Clay Adams) to flatten the dermal tissue for microscopic observation. Cremaster muscle (CM): animals were placed in a supine position on a specially designed plexiglass stage, and the right CM was prepared for intravital microscopy as described previously (Pemberton et al., 1993). Throughout the experiment, the CM was superfused with 37°C endotoxin-free, bicarbonate-buffered, oxygen-depleted saline solution.

Microvascular Observation

Animals were transferred to a microscope stage (IV-500, Mikron) and 10 ml per kilogram body weight of Ringer's lactate containing 2 mg/ml of the fluorescent dye rhodamine 6G (Molecular Probes) was injected intravenously. Visualization of labeled leukocytes was made by video-triggered stroboscopic epi-illumination using a Xenon Arc (Chadwick Helmuth) and a FITC filter set. Following rhodamine injection, video recordings of fluorescent leukocyte behavior in postcapillary and collecting venules (diameter 16–35 μM in ES and 20–60 μM in CM) were made using a 40× water immersion objective (Zeiss Achroplan, NA 0.75 ∞), a low-lag SIT camera (Dage MTI, Michigan City, IN), a time base generator (For-A), and a Hi-8 VCR (Sony). The preparations were used to compare leukocyte behavior in the absence of tissue irritation or inflammation in the ES and after mild surgery-induced tissue damage and during acute inflammation induced by the chemoattractant leukotriene B_4 (LTB_4) in the CM. We made 1 min recordings of fluorescent cells in four to seven CM venules during three consecutive 5 min intervals (control period). Immediately thereafter, the superfusion was replaced with 37°C buffer containing 10^{-7} M LTB_4. Following exposure to LTB_4, video recordings were made as described above. Video analysis for determining rolling fractions was performed as described previously (von Andrian et al., 1992). A PC-based interactive image analysis system for microcirculation research (Priess, 1988) was used to determine vessel diameter and axial length and velocities of individual rolling and noninteracting leukocytes. The mean blood flow velocity and wall shear rates were assessed as described previously (Ley and Gaehtgens, 1991).

Sampling and Determination of Peripheral Blood and Thioglycollate-Elicited Peritoneal Leukocyte Counts

Peripheral blood was obtained by retro-orbital sampling using heparinized capillary tubes and collected in 5 mM EDTA. Total leukocyte counts were determined on a Coulter counter after hemolysis of erythrocytes using Zap-oglobin II (Coulter). Differential counts were determined by examination of Wright–Giemsa-stained blood smears (Baxter). Platelet counts were done on a hemocytometer after lysis using the unopette microcirculation system (Becton Dickinson). Mice were injected intraperitoneally with 1 ml of 3% thioglycollate (Sigma), and peritoneal lavage was harvested as previously described (Mayadas et al., 1993). Total cells in the lavage were counted on a hemocytometer, and cytospins of the cells were stained with Wright–Giemsa. Cells were differentially counted to determine the percentage of neutrophils, eosinophils, and mononuclear cells.

Neutropenia was induced in mice by an intraperitoneal injection of cyclophosphamide (200 mg per kilogram body weight) (Sigma) (Voncken et al., 1995). Thioglycollate peritonitis was induced 72 hr

later as described above. Neutropenia was >98% as confirmed by leukocyte and differential counts in anti-coagulated blood.

Murine Neutrophil Isolation

PBN Isolation

Blood was collected in 5 mM EDTA and neutrophils were isolated by density centrifugation using Nim.2 neutrophil isolation medium according to the protocols described by the manufacturer (Cardinal Associates, Inc.). Approximately 2.8 ml of blood (pooled from three mice) was loaded onto each gradient (yields approximately 1.4×10^6 neutrophils) and referred to as one experiment. Each timepoint represents the average of three experiments.

Isolation of Thioglycollate-Elicited Neutrophils

Neutrophils were isolated from peritoneal lavage of 10.5 hr thioglycollate-treated mice by Percoll density gradient centrifugation, as previously described (Fadok et al., 1992). Neutrophils were present in density layers greater than or equal to 1.075. These cells were collected and placed in culture. Lavage from three mice was pooled and is referred to as one experiment. Each timepoint represents the average of eight experiments.

Assessment of Neutrophil Apoptosis

Isolated neutrophils were resuspended at 4×10^5 cells per milliliter in complete media (IMDM containing 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) and plated in 24-well dishes previously coated with Poly-HEMA (Sigma) to prevent adhesion. At various timepoints after culturing, approximately 4×10^4 cells were collected from each well for viability and apoptosis assessment. Total lavage leukocytes from 2 hr thioglycollate-treated mice were centrifuged and resuspended at 10^6 cells per milliliter of complete media and plated in coated wells. The percentages of mononuclear cells and neutrophils were comparable between wild-type and mutant samples. Percent viability was determined by trypan blue exclusion. Cytospin preparations were fixed in methanol, stained with Wright–Giemsa, and examined by oil-immersion light microscopy at a final magnification of 1000×. The percentage of apoptotic neutrophils was determined by counting the number of cells showing features associated with apoptosis (chromatin condensation and fragmented nuclei) as previously described (Savill et al., 1989). For all samples analyzed, 200–400 cells per slide were counted by the researcher without prior knowledge of the sample.

Flow cytometry on a FACScan (Becton Dickinson) was carried out on neutrophils stained with a MAb to CD16 (FcγRIII) or propidium iodide. Flow cytometric analysis of CD16 expression was performed on human neutrophils using FITC-conjugated mouse anti-human MAbs (Pharmingen) for 20 min on ice. Cells were washed, fixed in 4% paraformaldehyde, and subjected to flow cytometric analysis. We analyzed 5000 cells in each sample. For propidium iodide staining, 10^6 mouse or human neutrophils were pelleted and resuspended in 0.3 ml PBS. Ice-cold 100% ethanol (0.7 ml) was then added dropwise, and the sample was stored at 4°C for at least 2 hr. After washing, the cell pellets were resuspended in 0.25 ml of PBS; 0.25 ml of RNase A (1 mg/ml) and 0.5 ml of propidium iodide (100 μg/ml) (Sigma) were added, and samples were incubated at room temperature for 15 min with shaking. Samples were stored at 4°C in the dark until they were examined. We analyzed 10,000 cells in each sample.

DNA laddering in murine neutrophils was assessed as previously described (Lee et al., 1993). We loaded 2.5 μg of DNA per lane on a 1.8% TBE agarose gel containing ethidium bromide.

Assessment of Neutrophil Phagocytosis, Spreading, and Oxidative Burst

Complement-Opsonized Oil-Red-O Phagocytosis

Phagocytosis of serum-opsonized paraffin oil droplets was measured as detailed elsewhere (Arnaout et al., 1982). The method of opsonization leads to deposition of complement (C3) (Stossel, 1986). In brief, peritoneal lavage was collected from mice injected for 4 hr with thioglycollate. Total leukocytes from six wild-type or CD11b null mice were pooled, washed extensively with Tris saline, and resuspended at 4×10^7 cells per milliliter in Tris saline with 0.1% human serum albumin. We incubated 100 μl of the cell suspension with 175 μl of the serum-opsonized particles in a total volume of 550 μl in PBS (plus Ca and Mg) for 7 min, with or without 1 mM N-ethyl-maleimide (NEM), an inhibitor of phagocytosis. Cells were washed and the red dye was extracted using dioxane and phagocytosis quantified at OD_{525}. The rate of phagocytosis was then calculated as micrograms of Oil-red-O per 10^6 PMNs per minute. The same assay was used to assess human neutrophil phagocytosis; isolated neutrophils from normal or CGD patients were either left in buffer or pretreated (10 min on ice) with the function-blocking antibody to CD11b (44, IgG_{2a}; Arnaout et al., 1983) or 107 (IgG_1; Rieu et al., 1994) or the control antibody to CD11a (TS122, IgG_1; Mentzer et al., 1986). Antibodies were used at 1:50 dilution of ascites. Cells were then exposed to opsonized ORO for 3 min, washed, and then analyzed for apoptosis over time as described above. In some experiments, DPI (at 50 μM; Toronto Research Chemicals, Inc.) was added to normal PBN in a 7 min phagocytosis assay to determine the role of ROI in apoptosis. In experiments establishing that phagocytosis induces apoptosis, eight experiments were performed on neutrophil and serum isolated from different donors (range of 20%–90% neutrophil apoptosis observed after 2 hr in culture). In time course experiments with antibodies added, a single donor was used for consistency in the percentage of apoptosis observed after phagocytosis.

Neutrophil Spreading

Isolated mouse neutrophils were assayed for their ability to spread on a glass surface using a hemocytometer as previously described (Lee et al., 1993). Cells were loaded onto the hemocytometer, incubated for 5 min at room temperature, and scored for cell spreading under light microscopy.

Oxidative Burst

Peritoneal exudate was collected from 2 hr thioglycollate-treated wild-type and CD11b/CD18-deficient mice. The lavage was performed in 2 ml of buffer, and 200 μl of cell suspension was incubated with a phycoerythrin-labeled rat MAb to mouse Gr-1 (1:100) (Pharmingen) for 30 min at 4°C. Subsequently, 300 μl of PBS was added with 5 μM dichlorofluorescein diacetate (DCFA) (Molecular Probes) and incubated for 15 min at room temperature. Two-color cytofluorimetric analysis was performed on a Becton Dickinson FACScan to determine the mean DCF fluorescence associated with Gr-1-positive cells (Busciglio and Yankner, 1995).

The oxidative burst was also measured during ingestion of opsonized particles by including nitroblue tetrazolium (NBT, Sigma) (Baehner and Nathan, 1968) at a final concentration of 0.8 mg/ml. NBT reduction was then measured at the conclusion of the phagocytosis assay by measuring absorption of the dioxane extract at OD_{580} and correcting for the ORO absorption.

Statistics

Data are presented as average ± standard error of the mean. Statistical significance was assessed by unpaired Student's t test.

CGD Patients

Two patients with different genetic lesions in the NADPH oxidase were evaluated. One, a male, is lacking gp91 phox component of NADPH oxidase (P. Newburger, personal communication) and the second, a female, is suffering from a rare autosomal form of CG, lacking the p22 phox component of the oxidase (Dinauer et al., 1990). Both patients were receiving antibiotics on a chronic basis, but were otherwise healthy during the period of the study.

Acknowledgments

Correspondence should be addressed to T. N. M. We thank Dr. Robert Pytela (University of California, San Francisco) for providing cDNA for mouse CD11b, Drs. Abul Abbas and Ramzi Cotran (Department of Pathology, Harvard Medical School) for helpful discussions, Mr. Michael Goodman for mouse husbandry, and the patients who participated in this study. We thank Luk van Parijs for generously donating his time in operating the FACScan, Drs. Peter E. Newburger and Alan B. Esekowitz for providing the CGD patients, and Dr. Michael Gimbrone for critical reading of the manuscript. This work was supported by the National Multiple Sclerosis Society (T. N. M.), the American Heart Association (P. R.), and National Institutes of

Health grants NS33296 (T. N. M.), DK-48549 and PO150305 (M. A. A.), HL-48675 (U. V. A.), and 1PO30AR42689 (A. H. S).

Received August 29, 1996; revised September 30, 1996.

References

Abbas, A.K. (1996). Die and let live: eliminating dangerous lymphocytes. Cell *84*, 655–657.

Afford, S.C., Pongracz, J., Stockley, R.A., Crocker, J., and Burnett, D. (1992). The induction of human IL-6 of apoptosis in the promonocytic cell line U937 and human neutrophils. J. Biol. Chem. *267*, 21612–21616.

Anderson, D.C., and Springer, T.A. (1987). Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. Annu. Rev. Med. *38*, 175–194.

Anderson, D.C., Schmalsteig, F.C., Finegold, M.J., Hughes B.J., Rothlein, R., Miller, L.J., Kohl, S., Tosi, M.F., Jacobs, R.L., Waldrop, T.C., et al. (1985). The severe and moderate phenotypes of heritable Mac-1, LFA-1 deficiency: their quantitative definition and relation to leukocyte dysfunction and clinical features. J. Infect. Dis. *152*, 668–689.

Arbones, M.L., Ord, D.C., Ley, K., Ratech, H., Maynard-Curry, C., Otten, G., Capon, D.J., and Tedder, T.F. (1994). Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin (CD62L)-deficient mice. Immunity *1*, 247–260.

Arnaout, M.A. (1990a). Leukocyte adhesion molecule deficiency: its structural basis, pathophysiology and implications for modulating the inflammatory response. Immunol. Rev. *114*, 145–180.

Arnaout, M.A. (1990b). Structure and function of the leukocyte adhesion molecules CD11/CD18. Blood *75*, 1037–1050.

Arnaout, M.A., Pitt, J., Cohen, H.J., Melamed, J., Rosen, F.S., and Colten, H.R. (1982). Deficiency of a granulocyte-membrane glycoprotein (gp 150) in a boy with recurrent bacterial infections. N. Engl. J. Med. *306*, 693–699.

Arnaout, M.A., Todd, R.F., III, Dana, N., Melamed, J., Schlossman, S.F., and Colten, H.R. (1983). Inhibition of phagocytosis of complement C3- or immunoglobultin G-coated particles and of iC3b binding by monoclonal antibodies to a monocyte-granulocyte membrane glycoprotein (Mo1). J. Clin. Invest. *72*, 171–179.

Arnaout, M.A., Lanier, L.L., and Faller, D.V. (1988). Relative contribution of the leukocyte molecules Mo1, LFA-1, p150,95 (LeuM5) in adhesion of granulocytes and monocytes to vascular endothelium is tissue- and stimulus-specific. J. Cell Physiol. *137*, 305–309.

Babior, B.M. (1992). The respiratory burst oxidase. Adv. Enzymol. Relat. Areas Mol. Biol. *65*, 49–95.

Baehner, R.L., and Nathan, D.G. (1968). Quantitative nitroblue tetrazolium test in chronic granulomatous disease. New Eng. J. Med. *278*, 971–976.

Bullard, D.C., Scharffetter-Kochanek, K., McArthur, M.J., Chosay, J.G., McBride, M.E., Montgomery, C.A., and Beaudet, A.L. (1996). A polygenic mouse model of psoriasiform skin disease in CD18-deficient mice. Proc. Natl. Acad. Sci. USA *93*, 2116–2121.

Busciglio, J., and Yankner, B.A. (1995). Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons *in vitro*. Nature *378*, 776–779.

Buttke, T.M., and Sandstrom, P.A. (1994). Oxidative stress as a mediator of apoptosis. Immunol. Today *15*, 7–10.

Carlos, T.M., and Harlan, J.M. (1994). Leukocyte-endothelial adhesion molecules. Blood *84*, 2068–2101.

Chapes, S.K., and Haskill, S. (1983). Evidence for granulocyte-mediated macrophage activation after *C. parvum* immunization. Cell. Immunol. *75*, 367–377.

Colotta, F., Re, F., Polentarutti, N., Sozzani, S., and Mantovani, A. (1992). Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood *80*, 2012–2020.

Cox, G., Crossley, J., and Xing, Z. (1995). Macrophage engulfment of apoptotic neutrophils contributes to the resolution of acute pulmonary inflammation *in vivo*. Am. J. Resp. Cell Mol. Biol. *12*, 232–237.

Cross, A.R (1990). Inhibitors of the leukocyte superoxide generating oxidase: mechanisms of action and methods for their elucidation. Free Radic. Biol. Med. *8*, 71–93.

Dinauer, M.C., Pierce, E.A., Bruns, G.A., Curnulle, J.T., and Orkin, S.H. (1990). Human neutrophil cytochrome b light chain (p22-phox): gene structure, chromosomal location, and mutations in cytochrome-negative autosomal recessive chronic granulomatous disease. J. Clin. Invest. *86*, 1729–1737.

Dransfield, I., Buckle, A.-M., Savill, J.S., McDowall, A., Haslett, C., and Hogg, N. (1994). Neutrophil apoptosis is associated with a reduction in CD16(FcγRIII) expression. J. Immunol. *153*, 1254–1263.

Fadok, V.A., Savill, J.S., Haslett, C., Bratton, D.L., Doherty, D.E., Campbell, P.A., and Henson, P.M. (1992). Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. J. Immunol. *149*, 4029–4035.

Fallman, M., Andersson, R., and Andersson, T. (1993). Signaling properties of CR3 (CD11b/CD18) and CR1 (CD35) in relation to phagocytosis of complement-opsonized particles. J. Immunol. *151*, 330–338.

Firestein, G.S., Yeo, M., and Zvaifler, N.J. (1995). Apoptosis in rheumatoid arthritis synovium. J. Clin. Invest. *96*, 1631–1638.

Frenette, P.S., Mayadas, T.N., Rayburn, H., Hynes, R.O., and Wagner, D.D. (1996). Susceptibility to infection and altered hematopoiesis in mice deficient in both P- and E-selectin. Cell *84*, 563–574.

Gaboury, J.P., and Kubes, P. (1994). Reductions in physiologic shear rates lead to CD11/CD18-dependent, selectin-independent leukocyte rolling *in vivo*. Blood *83*, 345–350.

Giger, U., Boxer, L.A., Simpson, P.J., Lucchesi, B.R., and Todd, R.F., III (1987). Deficiency of leukocyte surface glycoproteins Mo1, LFA-1, and Leu-M5 in a dog with recurrent bacterial infections: an animal model. Blood *69*, 1622–1630.

Gottlieb, R.A., Giesing, H.A., Zhu, J.Y., Engler, R.L., and Babior, B.M. (1995). Cell acidification in apoptosis: granulocyte colony-stimulating factor delays programmed cell death in neutrophils by up-regulating the vacuolar H+-ATPase. Proc. Natl. Acad. Sci. USA *92*, 5965–5968.

Gresham, H.D., Graham, I.L., Anderson, D.C., and Brown, E.J. (1991). Leukocyte adhesion-deficient neutrophils fail to amplify phagocytic function in response to stimulation: evidence for CD11b/CD18-dependent and -independent mechanisms for phagocytosis. J. Clin. Invest. *88*, 588–597.

Grigg, J.M., Savill, J.S., Saraff, C., Haslett, C., and Silverman, M. (1991). Neutrophil apoptosis and clearance by macrophages in the lungs of neonates with pulmonary inflammation. Lancet *338*, 720–722.

Henderson, W.R., and Klebanoff, S.J. (1983). Leukotriene production and inactivation by normal, chronic granulomatous disease and myeloperoxidase-deficient neutrophils. J. Biol. Chem. *258*, 13522–13527.

Henson, P.M. (1991). Resolution of inflammation: a perspective. Chest (Suppl). *99*, 2S–5S.

Hockenbery, D.M., Oltvai, Z.N., Yin, X.-M., Milliman, C.L., and Korsmeyer, S.J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell *75*, 241–251.

Iwai, K., Miyawaki, T., Takizawa, T., Konno, A., Ohta, K., Yachie, A., Seki, H., and Taniguchi, N. (1994). Differential expression of bcl-2 and susceptibility to anti-Fas-mediated cell death in peripheral blood lymphocytes, monocytes, and neutrophils. Blood *84*, 1201–1208.

Jackson, S.H., Gallin, J.I., and Holland, S.M. (1995). The $p47_{phox}$ mouse knock-out model of chronic granulomatous disease. J. Exp. Med. *182*, 751–758.

Kehrli, M.E., Schmalsteig, F.C., Anderson, D.C., Van Der Maaten, M.J., Hughes, B.J., Ackermann, M.R., Wilhelmsen, C.L., Brown, G.B., Stevens, M.G., and Whetstone, C.A. (1990). Molecular definition of the bovine granulocytopathy syndrome: identification of deficiency of the Mac-1 (CD11b/CD18) glycoprotein. Am. J. Vet. Res. *51*, 1826–1836.

Lagasse, E., and Weissman, I.L. (1994). bcl-2 inhibits apoptosis of neutrophils but not their engulfment by macrophages. J. Exp. Med. *179*, 1047–1052.

Laird, P.W., Zijderveld, A., Linders, K., Rudnicki, M.A., Jaenisch, R., and Berns, A. (1991). Simplified mammalian DNA isolation procedure. Nucl. Acids Res. *19*, 4293.

Lee A., Whyte, M.K.B., and Haslett, C. (1993). Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. J. Leuk. Biol. *54*, 283–288.

Lewinsohn, D.M., Bargatze, R.F., and Butcher, E.C. (1987). Leukocyte-endothelial cell recognition: evidence of a common molecular mechanism shared by neutrophils, lymphocytes, and other leukocytes. J. Immunol. *138*, 4313–4321.

Ley, K., and Gaehtgens, P. (1991). Endothelial, not hemodynamic, differences are responsible for preferential leukocyte rolling in rat mesenteric venules. Circ. Res. *69*, 1034–1041.

Li, E., Bestor, T.H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell *69*, 915–926.

Liles, W.C., and Klebanoff, S.J. (1995). Regulation of apoptosis in neutrophils: Fas track to death? J. Immunol. *155*, 3289–3291.

Liles, W.C., Kiener, P.A., Ledbetter, J.A., Aruffo, A., and Klebanoff, S.J. (1996). Differential expression of Fas (CD95) and Fas ligand on normal human phagocytes: implications for regulation of apoptosis in neutrophils. J. Exp. Med. *184*, 429–440.

Mayadas, T.N., Johnson, R.C., Rayburn, H., Hynes, R.O., and Wagner, D.D. (1993). Leukocyte rolling and extravasation are severely compromised in P-selectin-deficient mice. Cell *74*, 541–554.

Mentzer, S.J., Faller, D.V., and Burakoff, S.J. (1986). Inerferon-γ induction of LFA-1 mediated homotypic adhesion of human monocytes. J. Immunol. *137*, 108–113.

Mulligan, M.S., Till, G.O., Smith, C.W., Anderson, D.C., Miyasaka, M., Tamatani, T., Todd, R.F., III, Issekutz, T.B., and Ward, P.A. (1994). Role of leukocyte adhesion molecules in lung and dermal vascular injury after thermal trauma of skin. Am. J. Pathol. *144*, 1008–1015.

Nathan, C., Srimal, S., Farber, C., Sanchez, E., Kabbash, L., Asch, A., Gailit, J., and Wright, S.D. (1989). Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. J. Cell Biol. *109*, 1341–1349.

Pemberton, M., Anderson, G., Vaclav, V., Justus, D.E., and Ross, G.D. (1993). Microvascular effects of complement blockade with soluble recombinant CR1 on ischemia/reperfusion injury of skeletal muscle. J. Immunol. *150*, 5104–5113.

Petty, H.R., and Todd, R.F., III (1993). Receptor-receptor interactions of complement receptor 3 in neutrophil membranes. J. Leuk. Biol. *54*, 492–494.

Pollock, J.D., Williams, D.A., Gifford, M.A.C., Li, L.L., Du, X., Fisherman, J., Orkin, S.H., Doerschuk, C.M., and Dinauer, M.C. (1995). Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocytes superoxide production. Nature Genet. *9*, 202–208.

Priess, A.R. (1988). A versatile video image analysis system for microcirculatory research. Int. J. Microcirc. Clin. Exp. *7*, 327–345.

Pytela, R. (1988). Amino acid sequence of the murine Mac-1 α chain reveals homology with the integrin family and an additional domain related to von Willebrand factor. EMBO J. *7*, 1371–1378.

Rieu, P., Ueda, T., Haruta, I., Sharma, C.P., and Arnaout, M.A. (1994). The A-domain of β2 integrin CR3 (CD11b/CD18) is a receptor for the hookworm-derived neutrophil adhesion inhibitor NIF. J. Cell Biol. *127*, 2081–2091.

Sanui, H., Yoshida, S.-i., Nomoto, K., Ohhara, R., and Adachi, Y. (1982). Peritoneal macrophages which phagocytose autologous polymorphonuclear leukocytes in guinea-pigs. I. Induction by irritants and microorganisms and inhibition by colchicine. Br. J. Exp. Pathol. *63*, 278–284.

Savill, J.S., Wyllie, A.H., Henson, J.E., Wolport, M.J., Henson, P.M., and Haslett, C. (1989). Macrophage phagocytosis of aging neutrophils in inflammation: programmed cell death in the neutrophil leads to its recognition by macrophages. J. Clin. Invest. *83*, 865–875.

Savill, J., Dransfield, I., Hogg, N., and Haslett, C. (1990). Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. Nature *343*, 170–173.

Schmits, R., Kundig, T.M., Baker, D.M., Shumaker, G., Simard, J.J.L., Duncan, G., Wakeham, A., Shahinian, A., van der Heiden, A., Bachmann, M.F., Ohashi, P.S., Mak, T.W., and Hickstein, D.D. (1996). LFA-1-deficient mice show normal CTL responses to virus but fail to reject immunogenic tumor. J. Exp. Med. *183*, 1415–1426.

Squier, M.K.T., Sehnert, A.J., and Cohen, J.J. (1995). Apoptosis in leukocytes. J. Leuk. Biol. *57*, 2–10.

Stossel, T.P. (1986). Oil-droplet method for measuring phagocytosis. Meth. Enzymol. *132*, 192–198.

Stuehr, D.J., Fasehun, O.A., Kwon, N.S., Gross, S.S., Gonzalez, J.A., Levi, R., and Nathan, C.F. (1991). Inhibition of macrophage and endothelial cell nitric oxide synthetase by diphenylene iodonium and its analogs. FASEB J. *5*, 98–103.

Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science *267*, 1456–1462.

Um, H.-D., Orenstein, J.M., and Wahl, S.M. (1996). Fas mediates apoptosis in human monocytes by a reactive oxygen intermediate dependent pathway. J. Immunol. *156*, 3469–3477.

von Andrian, U.H., Hansell, P., Chambers, J.D., Berger, E.M., Torres Filho, I., Butcher, E.C., and Arfors, K.E. (1992). L-selectin function is required for β2-integrin-mediated neutrophil adhesion at physiological shear rates *in vivo*. Am. J. Physiol. *263*, H1034–H1044.

von Andrian, U.H., Berger, E.M., Ramezani, L., Chambers, J.D., Ochs, H.D., Harlan, J.M., Paulson, J.C., Etzioni, J.C., and Arfors, K.-E. (1993). *In vivo* behavior of neutrophils from two patients with distinct inherited leukocyte adhesion deficiency syndromes. J. Clin. Invest. *91*, 2893–2897.

Voncken, J.W., van Schaick, H., Kaartinen, V., Deemer, K., Coates, T., Landing, B., Pattengale, P., Dorseil, O., Bokoch, G.M., Groffen, J., and Heisterkamp, N. (1995). Increased neutrophil respiratory burst in bcr-null mutants. Cell *80*, 719–728.

Watson, R.W.G., Redmond, H.P., Wang, J.H., Condron, C., and Bouchier-Hayes, D. (1996). Neutrophils undergo apoptosis following ingestion of *Escherichia coli*. J. Immunol. *156*, 3986–3992.

Whyte, M.K.B., Hardwick, S.J., Meagher, L.C., Savill, J.S., and Haslett, C. (1993). Transient elevations of cytosolic free calcium retard subsequent apoptosis of neutrophils *in vitro*. J. Clin. Invest. *92*, 446–455.

Wilson, R.W., Ballantyne, C.M., Wayne Smith, C., Montgomery, C., Bradley, A., O'Brien, W.E., and Beaudet, A.L. (1993). Gene targeting yields a CD18-mutant mouse for study of inflammation. J. Immunol. *151*, 1571–1578.

Witte, T., Dumoulin, F.-L., Gessner, J.E., Schubert, J., Gotze, O., Neumann, C., Todd, R.F., III, Deicher, H., and Schmidt, R.E. (1993). Defect of a complement receptor 3 epitope in a patient with systemic lupus erythematosus. J. Clin. Invest. *92*, 1181–1187.

Yousefi, S., Green, D.R., Blaser, K., and Simon, H.-U. (1994). Protein-tyrosine phosphorylation regulates apoptosis in human eosinophils and neutrophils. Proc. Natl. Acad. Sci. USA *91*, 10868–10872.

Zhou, M.J., and Brown, E.J. (1994). CR3 (Mac-1, αmβ2, CD11b/CD18) and Fc(γ)RIII cooperate in generation of a neutrophil respiratory burst: requirement for Fc(γ)RII and tyrosine phosphorylation. J. Cell Biol. *125*, 1407–1416.