Galectin-3 and soluble fibrinogen act in concert to modulate neutrophil activation and survival: involvement of alternative MAPK pathways

Gabriela C. Fernández[1,3], Juan M. Ilarregui[1,4], Carolina J. Rubel[1,3], Marta A. Toscano[4], Sonia A. Gómez[3], Macarena Beigier Bompadre[3], Martín A. Isturiz[3], Gabriel A. Rabinovich[4], and Marina S. Palermo[1,2,3]

[3]División Inmunología, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Buenos Aires, Argentina; [4]División Inmunogenética, Hospital de Clínicas "José de San Martín," Universidad de Buenos Aires, Buenos Aires, Argentina

Galectin-3 (Gal-3), a member of a family of highly conserved carbohydrate-binding proteins, has recently emerged as a novel cellular modulator at inflammatory foci. Here we investigated the effects of Gal-3 on central effector functions of human neutrophils, including phagocytosis, exocytosis of secretory granules, and survival. We examined the effects of Gal-3 alone or in combination with soluble fibrinogen (sFbg), an extracellular mediator that plays a key role during the early phase of the inflammatory response through binding to integrin receptors. In addition we evaluated the intracellular signals triggered by these mediators in human neutrophils. Human neutrophils incubated with recombinant Gal-3 alone increased their phagocytic activity and CD66 surface expression. In contrast to the known antiapoptotic effect of Gal-3 on many cellular types, Gal-3 enhanced PMN apoptotic rate. Preincubation with Gal-3 primed neutrophils to the effects of sFbg, resulting in a synergistic action on degranulation. On the other hand, Gal-3 and sFbg had opposite effects on PMN survival, and the simultaneous action of both agonists partially counteracted the proapoptotic effects of Gal-3. In addition, although sFbg induced its effects through the activation of the ERKs, Gal-3 led to p38 phosphorylation. Disruption of this signaling pathway abrogated Gal-3-mediated modulation of neutrophil degranulation, phagocytosis, and apoptosis. Together, our results support the notion that Gal-3 and sFbg are two physiological mediators present at inflammatory sites that activate different components of the MAPK pathway and could be acting in concert to modulate the functionality and life span of neutrophils.

Key words: apoptosis/galectins/inflammation/MAPK pathway/neutrophil activation

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[1]These authors contributed equally to this work.
[2]To whom correspondence should be addressed; e-mail: mspalermo@hematologia.anm.edu.ar

Introduction

Polymorphonuclear leukocytes (PMNs) circulate within the vasculature in a quiescent state, but during the early phase of an inflammatory response they receive different signals that are able to induce their recruitment to the inflammatory foci, as well as regulate several effector functions. The integrin family plays an early role in this process through binding to specific ligands (Hynes, 1992). In this regard, CD11b/CD18 is a β_2 integrin that serves as a receptor for fibrinogen, complement factor C3bi, fibrin, and collagens (Altieri *et al.*, 1990; Wright *et al.*, 1983) and modulates PMN locomotion, degranulation, phagocytosis, and apoptosis (Rubel *et al.*, 2001).

Galectins, a growing family of mammalian lectins highly conserved throughout animal evolution (Hirabayashi and Kasai, 1993), have recently attracted the attention of immunologists as novel regulators of inflammation (Almkvist and Karlsson, 2004; Rabinovich *et al.*, 2002, 2004). According to their structure, these β-galactoside-binding proteins have been classified into proto type (galectins-1, -2, -5, -7, -10, -11, -13, -14, and -15), chimera type (galectin-3), and tandem repeat type (galectins-4, -6, -8, -9, and -12) (Hirabayashi and Kasai, 1993; Rabinovich *et al.*, 2004). They share remarkable sequence similarities in the carbohydrate recognition domain, and many family members preferentially recognize galactose-containing saccharide ligands. Galectin-3 (Gal-3) has been reported to regulate different inflammatory cell types, and targeted mutation of *gal-3* gene results in an attenuated inflammatory response following immunological challenge (Colnot *et al.*, 1998; Hsu *et al.*, 2000). Gal-3 activates mast cells and basophils (Zuberi *et al.*, 1994), potentiates lipopolysaccharide-induced IL-1 production from monocytes (Jeng *et al.*, 1994), and induces monocyte-macrophage chemotaxis (Sano *et al.*, 2000) and phagocytosis (Sano *et al.*, 2003). Regarding the influence of Gal-3 on neutrophil physiology, this protein binds to the surface of PMN through CD66b (Almkvist *et al.*, 2001; Feuk-Lagerstedt *et al.*, 1999; Yamaoka *et al.*, 1995), induces cell aggregation (Timoshenko *et al.*, 2003), promotes PMN adhesion to laminin (Kuwabara and Liu, 1996), and promotes extravasation in response to infection (Sato *et al.*, 2002). Although it has been reported that Gal-3 activates the NADPH-oxidase in primed PMNs (Karlsson *et al.*, 1998; Yamaoka *et al.*, 1995), the effects of Gal-3 on central effector mechanisms during an inflammatory process, including phagocytosis, exocytosis of granule content (degranulation), and life span, have not yet been explored.

An inflammatory situation in vivo exposes the cell to a wide variety of mediators, either in a simultaneous or sequential manner, which could reciprocally potentiate or counteract their effects. Moreover, accumulating evidence has

raised the concept that the pattern of activation in several cells, including neutrophils, is dependent on the activation of alternative intracellular pathways. Thus the final outcome in the modulation of neutrophil activation and survival will depend on the balance of the intracellular signals triggered by those agents. Following this assumption, we investigated the intracellular signaling pathway elicited by Gal-3 in PMNs and analyzed the physiological effects derived from the interplay between Gal-3 and soluble fibrinogen (sFbg) as an integrin-dependent stimulus. SFbg activates human PMNs through a CD11b-dependent mechanism and was selected for our study on the basis of recent observations reporting the effect of this early inflammatory mediator in the up-regulation of CD66b surface expression, which acts as a major receptor for Gal-3 in PMNs (Almkvist *et al.*, 2001). In this context, we undertook this work to study the interplay between Gal-3 and sFbg in the modulation of neutrophil physiology and the intracellular signals triggered by these mediators.

Results

Effect of Gal-3 on neutrophil phagocytosis

Because PMNs are the main phagocytic cells and the effect of rhGal-3 on this central capacity of PMNs has not been previously studied, erythrophagocytosis was assayed using PMNs against optimally sensitized target cells. As shown in Figure 1, phagocytosis was significantly increased by 4 µg/ml of recombinant human Gal-3 (rhGal-3), and lower concentrations were not effective at modulating this function. Furthermore, when PMNs were previously incubated for 15 min with lactose (30 mM), rhGal-3 (4 µg/ml) was not able to affect basal values of phagocytosis, suggesting that the modu-

lation of phagocytosis by Gal-3 depends on the carbohydrate-binding properties of this protein. Because sFbg also increases Fc-dependent phagocytosis (Rubel *et al.*, 2001), we tested the effect of adding both stimuli together. Preincubation with either sFbg (6 µM) or rhGal-3 (4 µg/ml) did not induce a higher increase compared with each stimulus added separately (Figure 1), indicating no additive or synergistic action of rhGal-3 and sFbg on PMN-mediated phagocytosis.

Influence of Gal-3 on neutrophil degranulation

Neutrophil degranulation is an important inflammatory event secondary to PMN activation that can be studied phenotypically by measuring the up-regulation of the membrane marker CD66b. This molecule resides in the specific granules of resting PMNs and appears on the cell surface upon stimulation (Stocks *et al.*, 1995). As depicted in Figure 2,

Fig. 2. Effect of rhGal-3 on CD66b expression as an indicator of PMN degranulation. PMNs (2.5×10^6/ml) were incubated for 60 min at 37°C in medium (control), sFbg (6 µM), or 15 min with rhGal-3 (0.4 and 4 µg/ml). Then cells were centrifuged, washed, and stained with a specific anti-CD66b mAb as described in *Materials and methods*. (**A** and **B**) Representative histograms of CD66b expression after different treatments are shown. The shaded histograms represent staining with isotype controls. The thin lines represent a control indicating basal levels of CD66b expression in PMN. The *y* and *x* axes represent cell number and fluorescence intensity (FL-1) respectively. (**A**) PMNs were incubated with different concentrations of rhGal-3 and rhGal-3 (4 µg/ml) plus lactose (30 mM). (**B**) Hatched histograms represent PMNs incubated with sFbg (6 µM) alone, or in combination with rhGal-3 (4 µg/ml). (**C**) Each bar represents the mean ± SEM of eight independent experiments using cells from different donors in each experiment. PMNs incubated with rhGal-3 (4 µg/ml) in the presence of lactose (30 mM) is also shown. *$p < 0.01$ compared with control; #$p < 0.05$ compared to sFbg- or rhGal-3-treated cells.

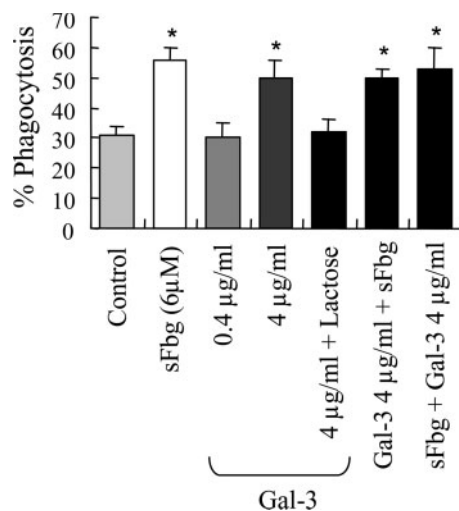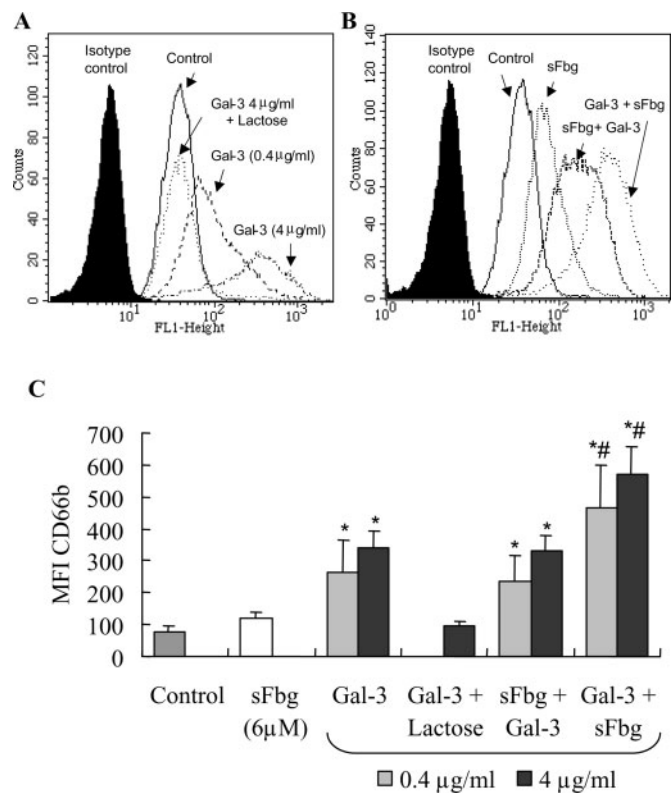**Fig. 1.** Effect of rhGal-3 on erythrophagocytosis. PMNs (2.5×10^6/ml) were incubated for 60 min at 37°C in medium (control), sFbg (6 µM), or 15 min with rhGal-3 (0.4 and 4 µg/ml), in the absence or presence of lactose (30 mM). After washing, phagocytosis assay was performed employing target cells optimally sensitized (IgG anti-sheep erythrocytes). Phagocytosis was evaluated after 30 min as described in *Materials and methods*. Data are expressed as the arithmetic mean ± SEM of five independent experiments using cells from different donors in each experiment. *$p < 0.05$ compared with control or Gal-3 in the presence of 30 mM lactose.

incubation for 15 min with rhGal-3 (0.4 and 4 µg/ml) was sufficient to induce a dose-dependent up-regulation of CD66b expression. Prolonged incubation up to 90 min did not show differences compared with 15-min exposure (data not shown). As a control, preincubation with 30 mM lactose prevented Gal-3-induced degranulation. In addition, pretreatment with sFbg (6 µM) for 60 min, which significantly increases CD66 membrane expression, did not modify the effects of rhGal-3. Conversely, 15 min preincubation with rhGal-3 synergistically enhanced the expression of CD66b induced by sFbg.

Modulation of neutrophil apoptosis by Gal-3 and sFbg

The role of Gal-3 on cell survival has been controversial. Intracellularly this protein has shown antiapoptotic activity in several cell types, including T cells and tumors (Moon *et al.*, 2001; Yang *et al.*, 1996), whereas exogenously added Gal-3 promoted apoptosis of T cell lines and primary T lymphocytes (Fukumori *et al.*, 2003). However, the effects of this protein on PMN survival have not yet been examined. On this versatile population, different activating stimuli, including sFbg, are able to inhibit the apoptotic program (Colotta *et al.*, 1992; Rubel *et al.*, 2001). On the other hand, other mediators such us TNF-α or IL-6 accelerate the PMN apoptotic rate (Afford *et al.*, 1992; Takeda *et al.*, 1993). Therefore, we investigated whether rhGal-3 could modulate neutrophil lifespan in addition to triggering PMN activation. Apoptosis was assessed by flow cytometry after 24 h of cell culture according to the frequency of subdiploid nuclei following permeabilization and propidium iodide (PI) staining. As

shown in Figure 3, preincubation with rhGal-3 (0.4 µg/ml) for 15 min enhanced the neutrophil apoptotic population from ~30% (control) to 50%. On the other hand, sFbg reduced PMN apoptosis to ~15%, as has been previously reported (Rubel *et al.*, 2001), and the combined treatment of rhGal-3 and sFbg, independent of the order of incubation, resulted in a partial blockade of the proapoptotic effect induced by exogenous Gal-3. This effect was dependent on the carbohydrate-binding properties of this protein because it was prevented by 30 mM lactose (Figure 3).

Intracellular signaling pathways triggered by Gal-3 in PMNs

In neutrophils, several inflammatory stimuli trigger mitogen-actived protein kinase (MAPK) phosphorylation (McLeish *et al.*, 1998; Nick *et al.*, 1997; Rane *et al.*, 1997). The observation that activation of these kinases play a pivotal role in regulating the lifespan of these inflammatory cells, together with our recent findings demonstrating that sFbg inhibits PMN apoptosis through an extracellular signal-regulated kinase (ERK)-dependent pathway (Rubel *et al.*, 2002), prompted us to investigate the activation of the MAPK pathway on incubation with rhGal-3. Phosphorylation of p38 and ERK following exposure of PMNs to rhGal-3 was investigated by flow cytometry and western blot analysis, respectively. As shown in Figure 4A, p38 phosphorylation was clearly induced 10 min after addition of rhGal-3, returning to basal levels after 15 min. On the other hand, western blot analysis using a specific anti-phosphoERK monoclonal antibody (mAb), revealed that rhGal-3 did not induce a significant phosphorylation of ERK1/2 in
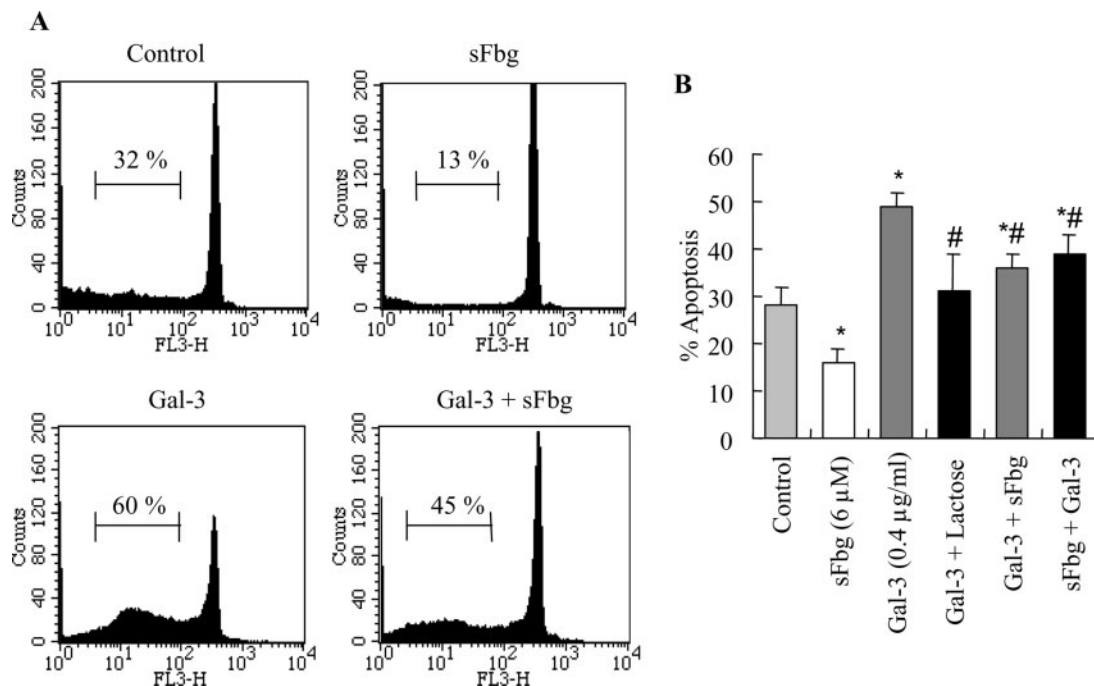

Fig. 3. Modulation of neutrophil apoptosis by rhGal-3. PMNs (2.5×10^6/ml) were incubated for 60 min at 37°C in medium (control), sFbg (6 µM), or 15 min with rhGal-3 (0.4 µg/ml), in the absence or presence of lactose (30 mM). All samples were washed and cultured for 24 h at 37°C. Then, the percentage of apoptotic cells was determined by PI staining. (**A**) Representative histograms showing the percentage (M1) nuclei with subdiploid DNA content after 24 h of incubation. (**B**) Results are expressed as the mean ± SEM of seven independent experiments using cells from different donors in each experiment. *$p < 0.01$ compared with control; #$p < 0.01$ compared with rhGal-3 treatment.
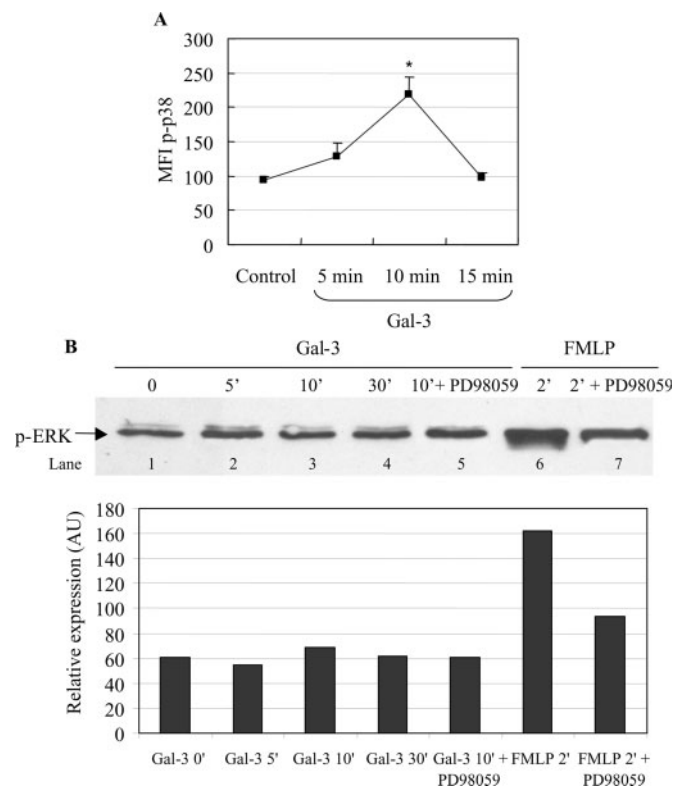
Fig. 4. rhGal-3 induces MAPK activation in PMNs. (**A**) PMNs
(2.5×10^6/ml) were incubated at 37°C with medium or rhGal-3 (0.4 µg/ml)
for the time periods indicated. Then cells were centrifuged, washed, and
stained with a specific anti-phospho-p38 mAb as described in *Materials
and methods*. Data are the mean ± SEM of five independent experiments
using cells from different donors in each experiments. *$p < 0.05$ compared
with controls. (**B**) Western blot analysis of ERK1/2 phosphorylation.
PMNs (2.5×10^6/ml) were incubated at 37°C with medium alone (lane 1)
or rhGal-3 (0.4 µg/ml) for the indicated time periods (lanes 2–5). As
controls, PMNs were incubated with FMLP (10^{-7} M) (lane 6) or FMLP
plus the PD98059 inhibitor (lane 7). Cells were then centrifuged, washed,
and lysed in the presence of a protease inhibitor cocktail and analyzed
by western blot analysis as described in *Materials and methods*.
Densitometric analysis of the intensity of each band (expressed as
arbitrary units) is also shown (lower panel).

periods ranging from 5 to 30 min (Figure 4B, lanes 2 to 5).
As a positive control of ERK activation, PMNs from the
same donor were incubated 2 min with N-formyl-methionyl-
leveyl-phenylelanine (FMLP) (10^{-7} M) (Figure 4B, lane 6).
Furthermore, preincubation with PD98059, an inhibitor of
ERK1/2 phosphorylation, abolished phosphorylation of
ERK, confirming the specificity of the antibody and effec-
tiveness of the inhibitor (Figure 4, lane 7). In contrast to
these observations, we previously demonstrated the ability
of sFbg to activate ERK1/2 and not p38 in a time-dependent
manner (Rubel *et al.*, 2002), suggesting that Gal-3 and
sFbg activate different intracellular pathways to modu-
late neutrophil activation and survival.

Modulation of Gal-3 effects by MAPK inhibitors

To determine the involvement of the MAPK cascade in
Gal-3-induced modulation of PMN functionality, we invest-
igated the effects of pharmacological inhibitors of ERK

and p38 MAPK pathways in Gal-3-induced degranulation,
enhancement of erythrophagocytosis, and apoptosis rate of
PMN. For this purpose, we used PD98059, an inhibitor of
MEK1 and MEK2 kinases, which are responsible for MAPK
ERK1/2 phosphorylation (Alessi *et al.*, 1995), and SB203580,
a phamacological inhibitor of p38 MAPK (Cuenda *et al.*,
1995). The effects of these inhibitors on sFbg-induced
increased degranulation and phagocytosis and decreased
apoptosis were previously studied (Rubel *et al.*, 2002).
Briefly, the effects of sFbg on those functions were shown
to be abolished by PD98059 and not modified by SB203580
(MFI CD66 expression: sFbg = 185 ± 25, sFbg + PD98059
= 78 ± 9 ($p < 0.005$); % phagocytosis: sFbg = 62 ± 4, sFbg +
PD98059 = 40 ± 2 ($p < 0.05$); % apoptosis: sFbg = 18±2,
sFbg + PD98059 = 40 ± 4 ($p < 0.005$). As shown in Figure 5
(A, B, and C), 30 µM SB203580 significantly decreased the
effects of rhGal-3 on phagocytosis, apoptosis, and CD66b
membrane expression. On the other hand, 50 µM PD98059
was not able to modify rhGal-3 effects, except CD66b
exocytosis, which was partially blocked by this pharmaco-
logical inhibitor. The ability of PD98059 to inhibit ERK
activation was confirmed by western blot analysis on
FMLP-activated PMN (Figure 4B, lane 7).

Taken together, these data indicate that the p38 MAPK
pathway is critically involved in Gal-3-induced modulation
of PMN functionality and survival.

Discussion

In this study we observed novel effects of exogenously
added Gal-3 on PMN that contributes to the activation
pattern of circulating neutrophils. Gal-3 induces an
increase in antibody-dependent erythrophagocytosis, mod-
ulates PMN degranulation as shown by up-regulated sur-
face expression of CD66, and influences PMN survival.
Gal-3-mediated modulation of PMN functions was depen-
dent on the carbohydrate-binding activity of this lectin,
because preincubation with lactose was able to prevent
these effects.

The observation that Gal-3 induces PMN activation is
consistent with the ability of this protein to stimulate super-
oxide production (Karlsson *et al.*, 1998; Yamaoka *et al.*,
1995), to enhance adhesiveness to extracellular matrix pro-
teins (Kuwabara and Liu, 1996) and to promote extravasa-
tion in response to infectious agents (Sato *et al.*, 2002).
Several studies have reported the requirement of previous
priming with bacterial derivates to observe Gal-3-mediated
effects on PMN (Faldt *et al.*, 2001; Karlsson *et al.*, 1998).

Although we have demonstrated that Gal-3 has direct
effects on purified PMNs, it is possible that the purification
procedure and fetal bovine serum (FBS)-containing media
could prime PMNs, as has been previously demonstrated
(Haslett *et al.*, 1985; Rubel *et al.*, 2001). On the other hand,
pretreatment with Gal-3 markedly enhanced the effects of
sFbg on degranulation, whereas preincubation with sFbg
did not modify the effects of Gal-3 on PMN response.

Although the molecular mechanisms responsible for the
induction of this sensitized state are still unclear, a review of
the current literature makes it evident that several signals
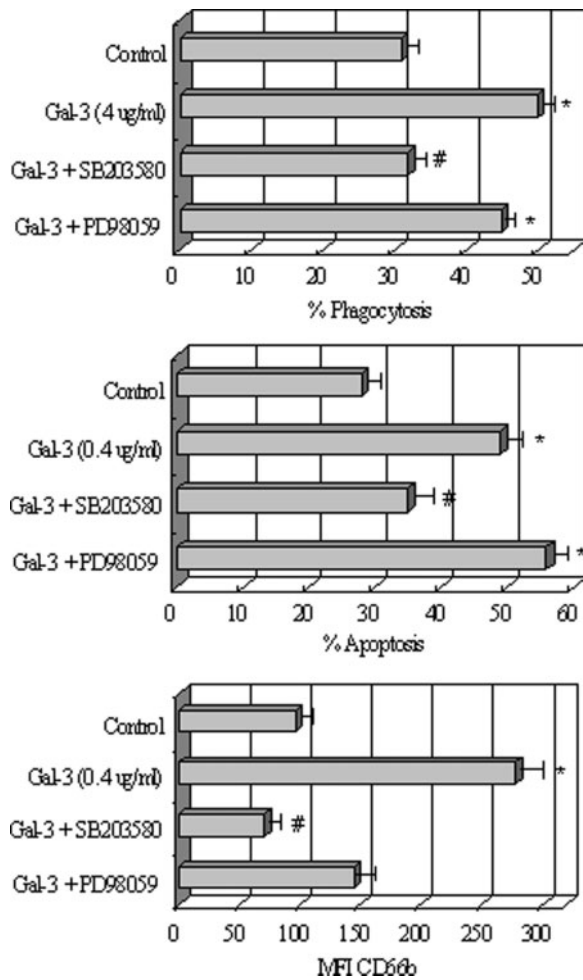work in concert to modulate PMN physiology (Hallett and

A

Control

Gal-3 (4 ug/ml) *

Gal-3 + SB203580 #

Gal-3 + PD98059 *

0   10   20   30   40   50

% Phagocytosis

B

Control

Gal-3 (0.4 ug/ml) *

Gal-3 + SB203580 #

Gal-3 + PD98059 *

0   10   20   30   40   50   60

% Apoptosis

C

Control

Gal-3 (0.4 ug/ml) *

Gal-3 + SB203580 #

Gal-3 + PD98059

0   50   100   150   200   250   300

MFI CD66b

Fig. 5. Gal-3 mediated modulation of PMN physiology by MAPK inhibitors. PMNs (2.5×10^6/ml) were preincubated 30 min with medium (control), PD98059 (50 µM), or SB203580 (30 µM), followed by stimulation for 15 min with the indicated concentration of rhGal-3 at 37°C. (**A**) Erythrophagocytosis. After washing, phagocytosis assay was performed and evaluated as described in *Materials and methods*. Data are expressed as the arithmetic mean ± SEM of five independent experiments using cells of different donors in each experiment. *$p < 0.01$ compared with control; #$p < 0.01$ compared with rhGal-3. (**B**) Apoptosis. All samples were washed and cultured for 24 h at 37°C. Then the percentage of apoptotic cells was determined by PI staining. Results are expressed as the mean ± SEM of five independent experiments using cells from different donors in each experiment. *$p < 0.01$ compared with control; #$p < 0.01$ compared with rhGal-3 treatment. (**C**) CD66 membrane expression. Cells were washed and stained with a specific anti-CD66b mAb as described in *Materials and methods*. Data represent the mean ± SEM of MFI from four different donors. *$p < 0.001$ compared with control; #$p < 0.001$ and **$p < 0.05$ compared with rhGal-3 treatment.

Lloyds, 1995). Suggested mechanisms include signaling events, such as tyrosine phosphorylation, increase in intracellular free calcium, and exposure of new receptors. In this regard, it has been previously documented that Gal-3 increases CD11b-dependent adhesion to extracellular matrix proteins (Matarrese *et al.*, 2000; Ochieng *et al.*, 1998). Moreover, cross-linking of CD66, which has been postulated to be a major Gal-3 receptor in PMNs (Feuk-Lagerstedt *et al.*, 1999; Kuwabara and Liu, 1996), has been shown to augment adhesion to fibrinogen (Stocks *et al.*, 1996). Thus

an alternative explanation for the synergistic effects of Gal-3 and sFbg may be that Gal-3 increases avidity of CD11b, which has been postulated to act as a receptor for sFbg on the cell surface of PMNs (Altieri *et al.*, 1990; Wright *et al.*, 1988). However, we cannot rule out other potential mechanisms, which might be also operating to increase the responsiveness of PMN to fibrinogen.

Although Gal-3 sensitized PMNs to sFbg effects, pretreatment with sFbg did not affect PMN response to Gal-3 effects. Almkvist and colleagues (2001) demonstrated that lipopolysaccharide primes PMNs to Gal-3 effects through CD66b mobilization. In our system, pretreatment with sFbg was not effective at modulating responsiveness of PMN to Gal-3, although sFbg successfully increased CD66b expression.

A remarkable set of data from the present work is the observation that Gal-3, broadly described as a molecule with antiapoptotic and proliferative effects on cells of different origin (Inohara *et al.*, 1998; Moon *et al.*, 2001; Yang *et al.*, 1996), has proapoptotic activity when added to PMNs. In this sense, Gal-3 exogenously added to human lung fibroblastic cells stimulated DNA synthesis as well as cell proliferation (Inohara *et al.*, 1998); Gal-3 expression has been associated with neoplastic progression and metastatic potential on several tumor cells (Takenaka *et al.*, 2004). Furthermore, in synovial mononuclear leukocytes from arthritic patients, the up-regulation of Gal-3 expression has been associated with the inhibition of apoptosis (Harjacek *et al.*, 2001). Moreover, Gal-3 has been demonstrated to be also involved in B cell differentiation and survival (Acosta-Rodriguez *et al.*, 2004; Hoyer *et al.*, 2004). In contrast, we have demonstrated that Gal-3, exogenously added to PMNs at low doses of 0.4 µg/ml, induces a strong proapoptotic signal. In this sense, a recent report suggests that extracellular Gal-3 can act as a proapototic signal in human T leukemia cell lines, human peripheral blood mononuclear cells, and activated mouse T cells (Fukumori *et al.*, 2003). Thus the assumption that Gal-3 can act either as an anti- or proapoptotic molecule is worthwhile to be discussed in terms of different variables, such as the target cell type, subcellular localization of this protein, activation state of the target cells, and the balance of intracellular signals that can potentiate or counteract its effects.

The MAPK cascade is known to participate in multiple cellular functions, such as degranulation, locomotion, proliferation, differentiation, and survival (Lewis *et al.*, 1998). The MAPKs are phosphorylated on threonine-tyrosine residues by distinct MAPK kinases. ERK1/2 are activated by a variety of growth factors and play a critical role in mitogenesis (Robinson and Cobb, 1997). JNK and p38 are typically activated by cellular stress or proinflammatory cytokines that are known to induce cell death (Raingeaud *et al.*, 1995), although recent studies have also demonstrated their activation by hematopoietic growth factors (Foltz *et al.*, 1997).

For a better understanding of the mechanisms involved in neutrophil activation, it is of central interest to investigate the functional relevance of signaling molecules. In the present work, we showed for the first time that Gal-3 stimulates p38 MAPK activity in a time-dependent manner with a maximum activity at 10 min, whereas there is no significant

ERK activation at periods ranging from 5 to 30 min. The role of MAPKs activation in the modulation of PMN functionality by Gal-3 was examined through the use of specific pharmacological inhibitors: (1) PD098059, which prevents ERK activation (Alessi *et al.*, 1995), and (2) SB203580, which belongs to a group of related compounds involved in p38 MAPK inhibition (Cuenda *et al.*, 1995). Inhibition of the p38 MAPK pathway completely blocked Gal-3-mediated stimulation of phagocytosis, apoptosis, and CD66b exocytosis. However, ERK inhibition had no effect on phagocytosis and modulation of apoptosis but partially inhibited Gal-3-mediated effects on CD66 exocytosis. In contrast, we have recently reported that sFbg induces ERK but not p38 phosphorylation (Rubel *et al.*, 2002), and the translocation of NF-kB to the nucleus through a mechanism that involves ERK activation (Rubel *et al.*, 2003). Because sFbg also activates phagocytosis and CD66b exocytosis but inhibits apoptosis, we can conclude that activation of both ERK1/2 and p38 cascades leads to stimulation of the phagocytic and degranulation activities of PMN. However, although ERK1/2 activation prevents apoptosis, p38 seems to play a key role in eliciting the neutrophil cell death program similarly to other cellular types (Frasch *et al.*, 1998; Waterman and Sha'afi, 1995). Therefore, the functional modulation of PMNs by Gal-3 and sFbg acting separately or in combination may reflect the signaling pathways activated by each of them. Moreover, the final outcome of the combined treatment on PMN survival independently of the sequence of addition of these proinflammatory agents reflects the intracellular balance of two opposite signals, though favoring the proapoptotic pathway. In this regard, the high sensitivity of PMNs to proapoptotic signals would be a protective mechanism to avoid healthy tissue damage provoked by an augmented release of oxygen metabolites and proteases.

In conclusion, sFbg and Gal-3 are two physiological mediators present at inflammation sites that may modulate the functionality and life span of neutrophils through the activation of differential intracellular signaling pathways.

Materials and methods

Reagents and mAbs

Acridine orange, ethidium bromide, PI, aprotinin, leupeptin, pepstatin A, PMA, diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, and lactose were obtained from Sigma (St. Louis, MO); human fibrinogen was obtained from Calbiochem (Buenos Aires, Argentina). MAPK inhibitors SB203580 and PD98059 were purchased from Calbiochem-Novabiochem (La Jolla, CA). Anti-phospho-ERK1/2 mAb, anti-phospho-p38 mAb, anti-p38 polyclonal antibody, and anti-ERK-2 polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate (FITC)-conjugated mouse mAb 80H3 (IgG$_1$) against human CD66b and FITC-conjugated isotype control (mouse IgG$_1$) were obtained from Caltag Laboratories (Burlingame, CA). rhGal-3 was kindly provided by Prof. J. Hirabayashi and K.I. Kasai (Teikyo University, Japan) or produced essentially as described

(Hirabayashi *et al.*, 1989). Briefly, DNA fragments were amplified by polymerase chain reaction using cloned cDNA as a template. The amplified fragments were ligated to pET21a. Generated expression vectors were used to transform *Escherichia coli* BL21 (DE3) cells. The recombinant protein was purified by affinity chromatography on asialofetuin-Sepharose 4B, which was prepared according to Hirabayashi and Kasai (1984). Lipopolysaccharide content of the purified protein was less than 60 ng/mg protein, determined with a colorimetric endotoxin determination reagent (Pyrodick, Seikagaku, Tokyo, Japan).

Blood samples

Blood samples were obtained from healthy volunteer donors who had taken no medication for at least 10 days before the day of sampling. Blood was obtained by venipuncture of the forearm vein and was drawn directly into citrated plastic tubes.

Neutrophil isolation

Neutrophils were isolated by Ficoll-Hypaque gradient centrifugation (Ficoll Pharmacia, Uppsala; Hypaque, Wintthrop Products, Buenos Aires) and dextran sedimentation. Contaminating erythrocytes were removed by hypotonic lysis. After washing, cells (> 96% neutrophils on May Grunwald/Giemsa-stained Cyto-preps) were suspended in RPMI 1640 supplemented with 1% heat-inactivated FBS.

Degranulation of neutrophils

Expression of the surface marker CD66b on the neutrophil surface was used as an indicator of degranulation of secondary granules (Niessen and Verhoeven, 1992). After preincubation at 37°C during different periods with or without sFbg, neutrophils were washed at 4°C with phosphate buffered saline (PBS) supplemented with 1% FBS and incubated with a mAb against CD66b. Control of isotype-matched antibody was assayed in parallel. Cells were then washed with cool PBS supplemented with 1% FBS and resuspended in 0.3 ml of ISOFLOW (International Link, Buenos Aires). Fluorescence was measured with a FACS cytometer (Becton Dickinson, CA). The analysis was carried out on 20,000 events on each sample by using the Cell Quest program.

Antibody-mediated erythrophagocytosis

Erythrophagocytosis was performed as previously described (Gresham *et al.*, 1988). Briefly, after preincubation with saline, rhGal-3, or sFbg for 1 h at 37°C, human neutrophils (50 μl, 7×10^6/ml) were washed and mixed with sheep erythrocytes (50 μl, 3% v/v) previously sensitized with subagglutinating amounts (200 μg) of rabbit IgG anti-sheep erythrocytes (Sigma). After incubation for 30 min at 37°C in 5% CO_2–95% humidified air, the non-ingested erythrocytes were lysed by hypotonic shock. Percentage of phagocytic neutrophils was evaluated by microscopic examination. At least 100 cells were scored in each sample. No phagocytosis was detected when neutrophils were incubated with unsensitized erythrocytes.

*Assessment of neutrophil apoptosis by PI staining
and flow cytometry*

The frequency of neutrophils with subdiploid DNA content was determined using a modification of the Nicoletti's protocol (Nicoletti *et al.*, 1991). Briefly, cell pellets containing 2.5×10^6 neutrophils were suspended in 400 μl hypotonic fluorochrome solution (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100) and incubated for 2 h at 4°C. The red fluorescence (PI staining) of individual nuclei was measured using a FACS flow cytometer (Becton Dickinson). The forward scatter and side scatter of particles were simultaneously measured. Cell debris was excluded from analysis by appropriately raising the forward-scattered threshold. The red fluorescence peak of neutrophils with normal (diploid) DNA content was set at channel 250 in the logarithmic mode. Apoptotic cell nuclei emitted fluorescence in channels 4 through 200.

Flow cytometric assays for p38 phosphorylation

One hundred microliters of purified neutrophils (10^7 PMN/ml) were processed following the instructions of Fix & Perm Cell Permeabilization Kit (Caltag Laboratories). Briefly, cells were fixed in suspension, washed, and permeabilized in the presence of the specific conjugated antibody. This procedure gives antibodies access to intracellular structures and does not affect the morphological scatter characteristics of the cells (Knapp *et al.*, 1994). Fluorescence was measured in a FACS cytometer, and the analysis was carried out on 20,000 events in each sample by using the Cell Quest program.

Western blot analysis

For each condition, PMNs at 2×10^7 cells/ml were used. Following different experimental treatments, cells were washed with PBS and lysed by incubation on ice for 20 min in 0.5 ml 100 mM Tris–HCl (pH 8.0), 100 mM NaCl, 2 mM ethylenediamine tetra-acetic acid, 1% Nonidet P-40 (RIPA buffer), 1 mM Na_3VO_4, 50 mM NaF, 0.3 U/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin and pepstatin A. Lysates were then centrifuged for 15 min at $14,000 \times g$. Protein concentrations were determined using the micro-VCA protein assay (Pierce, Rockford, IL). Samples were then prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions; this was performed on 10% minigels using standard Tris-glycine buffers. Proteins were transferred to polyvinylidene difluoride membranes (BioRad, Hercules, CA) for 1 h at 300 mA and blocked with PBS containing 3% nonfat dry milk for 1 h. Membranes were then probed with primary antibody in PBS 0.4% bovine serum albumin (anti-phospho ERK; 0.4 μg/ml) overnight. After washing three times with PBS 0.2% Tween 20, blots were incubated for 1 h with a horseradish peroxidase–conjugated goat anti-mouse or anti-rabbit IgG (Amersham, Aylesbury, U.K.). Immunoreactivity was detected using the ECL western blot detection kit (Amersham).

Statistical analysis

Results are expressed as the mean ± SEM. Statistical analysis of the data was performed using a nonparametric paired Mann-Whitney test. *p*-Values less than 0.05 were considered statistically significant.

Abbreviations

ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FMLP, N-formyl-methionyl-leveyl-phenylelanine; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; PBS, phosphate buffered saline; PI, propidium iodide; PMN, polymorphonuclear neutrophil; rhGal-3, recombinant human galectin-3; sFbg, soluble fibrinogen.

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