

The β -galactoside binding immunomodulatory lectin galectin-3 reverses the desensitized state induced in neutrophils by the chemotactic peptide f-Met-Leu-Phe: role of reactive oxygen species generated by the NADPH-oxidase and inactivation of the agonist

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Received on May 27, 2008; revised on August 19, 2008; accepted on August 19, 2008

Neutrophils interacting with a chemoattractant gradually become nonresponsive to further stimulation by the same agonist, a process known as desensitization. Receptor desensitization is a highly regulated process that involves different mechanisms depending on which receptor–ligand pair that is studied. Galectin-3, a member of a large family of β -galactoside-binding lectins, has been suggested to be a regulator of the inflammatory process, augmenting or directly triggering the neutrophil functional repertoire. We show here that the desensitized state of neutrophils interacting with the chemotactic peptide fMLF is broken by galectin-3 and that this is achieved through an oxygen radical-mediated inactivation of the chemoattractant. The effect was inhibited by the competitor lactose and required the affinity of galectin-3 for *N*-acetyllactosamine, a saccharide typically found on cell surface glycoproteins. The latter was shown using a galectin-3 mutant that lacked *N*-acetyllactosamine binding activity, and this protein was not active. The mechanism behind the inactivation of the chemoattractant was found to depend on the ability of galectin-3 to induce a neutrophil generation/secretion of reactive oxygen species which in combined action with myeloperoxidase inactivated the peptides.

Keywords: formylpeptide receptors/hydrogen peroxide/lectin/myeloperoxidase/oxidants

Introduction

The human defense toward microorganisms is largely dependent on the armory of neutrophil granulocytes, cells that are triggered to generate potent bactericidal (and sometimes tissue destructive) metabolites at sites of infection/inflammation (Dahlgren and Karlsson 1999; Gallin and Snyderman 1999). Neutrophil triggering may be mediated by a large number of

exogenous proinflammatory mediators (Durstin et al. 1994; Gallin and Snyderman 1999; Fu et al. 2006) or by endogenous agonists such as IL-8 (IL-8), a cytokine of the CXC family (Baggiolini et al. 1992), split products of complement components (i.e., C5a) (Goldstein et al. 1973) or other proteins (e.g., annexins) (Karlsson et al. 2005). We have recently added a new group of proteins, the galectins, to the list of endogenous inflammatory mediators that have the ability to activate neutrophils (Karlsson et al. 1998; Almkvist et al. 2002; Almkvist and Karlsson 2004; Carlsson et al. 2007). The galectin family of proteins is defined by their β -galactoside-binding capacity mediated by conserved sequence elements located in their carbohydrate-recognition domain (CRD) (Barondes et al. 1994; Dumic et al. 2006). The galectins promote inflammation (Liu and Hsu 2007) and they have been suggested to be involved in several parts of the innate immune system (Chen et al. 2005; Liu 2005; Dumic et al. 2006), including the recruitment of phagocytes and the recognition and killing of bacteria (Almkvist and Karlsson 2004; Chen et al. 2006; Barrionuevo et al. 2007; Farnworth et al. 2008). Galectin-3 is produced by many cells including neutrophils, but the main producers are macrophages (Almkvist and Karlsson 2004; Liu 2005; Dumic et al. 2006). The protein contains one CRD linked by a collagenase-sensitive domain to an N-terminal aggregating domain that enables the molecule to form oligomers (Barondes et al. 1994; Seetharaman et al. 1998; Ahmad et al. 2004). This lectin activates the superoxide generating NADPH-oxidase of human neutrophils, provided that the cells have first been primed (Almkvist and Karlsson 2004; Karlsson et al. 1998). The priming phenomenon has been described for many settings in neutrophil activation processes (Karlsson et al. 1998; Almkvist et al. 2001, 2004), and we have suggested that the priming mechanism in relation to the galectins involves mobilization of receptor storing intracellular granules (Almkvist et al. 2004). The membrane exposed receptor involved has been suggested to be a CEACAM (also known as CD66), stored in the mobilizable gelatinase/specific granules (Borregaard and Cowland 1997; Feuk-Lagerstedt et al. 1999) but other cell surface receptors may also be involved (Hernandez et al. 2006; Nieminen et al. 2007).

A large number of chemoattractant receptors have been characterized during the last 20-year period, all being seven-transmembrane-spanning receptors that are associated with a signaling heterotrimeric G-protein (Murphy 1994, 1997). The formyl peptide receptor (FPR1) was the first neutrophil G-protein coupled receptor (GPCR) to be cloned and sequenced (Boulay et al. 1990a; Boulay et al. 1990b). FPR1 is a high-affinity pattern-recognition receptor with the ability to track bacteria releasing formylated peptides (Schiffmann et al. 1975). Binding of formylated peptides by FPR1, e.g., the prototype chemoattractant formylmethionyl-leucyl-phenylalanine

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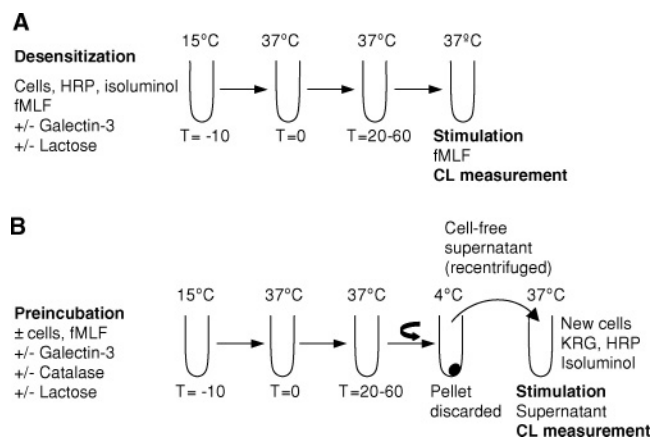


Fig. 1. Experimental setup for neutrophil desensitization and inactivation of chemoattractants. The two different techniques were used to determine desensitization (**A**) and inactivation of chemoattractants (**B**) following the interaction between neutrophils and the agonists. In the first type of experiments (**A**), the oxygen radical measuring chemicals (isoluminol and HRP) were present during the whole desensitization/inactivation protocol, and the same batch of cells were triggered with the agonist at two different occasions. In the second type of experiments (**B**), one cell sample was used for the inactivation of an agonist, and new batch of cells were triggered with cell-free supernatant obtained after the inactivation step.

(fMLF), activates a number of neutrophil functions. However, when the cells encounter the increasing concentration of these peptides they gradually become nonresponsive to further stimulation by the same agonist. This process, known as homologous receptor desensitization (Ali et al. 1999), is important to limit or terminate the response to higher concentrations of an attractant. The mechanism responsible for desensitization differs for different GPCRs and may involve phosphorylation of the occupied receptors by specific kinases, β -arrestin binding, phosphorylation of nonoccupied receptors, or physical coupling of the occupied receptor to the cytoskeleton (Klotz and Jesaitis 1994; Miller and Falke 2004).

In this study we show that galectin-3 can reverse the desensitized state of the neutrophil FPR1. This breakage of desensitization was achieved through an inactivation of the agonist (the peptide chemoattractant), and although no classical oxygen radical burst was induced during inactivation, the inhibitory activity was shown to be due to a combined action of released reactive oxygen species (ROS) and the azurophil granule protein myeloperoxidase (MPO).

Results

Neutrophil desensitization and the effect of galectin-3

It is well known that neutrophils challenged with the chemotactic peptide fMLF at 37°C are rapidly activated with regard to their capacity to generate superoxide anion, with peak production within the first minute. This constitutes a classical oxidative burst. Neutrophils that were incubated with fMLF at 15°C and then transferred to 37°C did not respond with an oxidative burst (data not shown). In addition, these cells did not respond to further fMLF stimulation (see Figure 1A), and the neutrophils remained nonresponsive (desensitized) for at least an hour (Figure 2, inset). Neutrophils that were incubated with fMLF

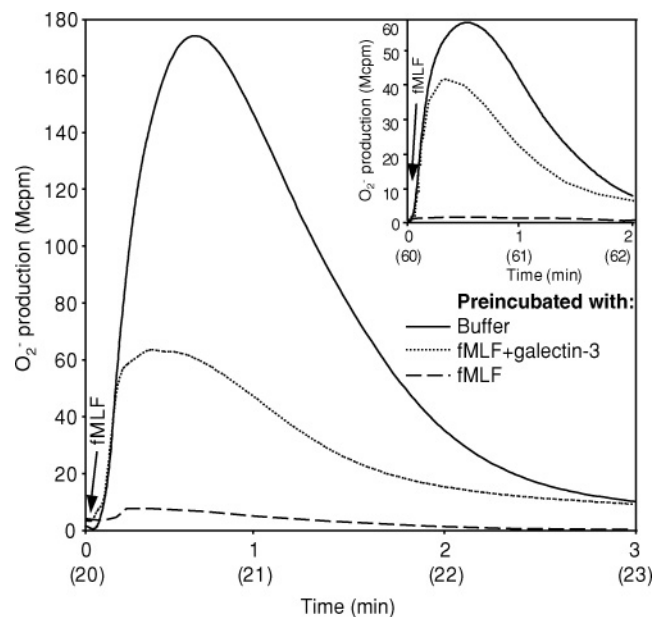


Fig. 2. Neutrophil desensitization and the effect of galectin-3. Neutrophils ($10^6/\text{mL}$) were incubated at 15°C for 10 min together with fMLF (10^{-7} M) in the presence or absence of galectin-3 ($20 \mu\text{g}/\text{mL}$) as described in Figure 1A. The samples also contained isoluminol and HRP to allow a measurement of radicals produced. The cell samples were transferred to 37°C and after a 20 min incubation period, a new dose of fMLF was added and the release of superoxide was recorded. A buffer control sample in which the cells were incubated at 15°C without any fMLF/galectin-3 is also shown. Neutrophils incubated with fMLF alone for 60 min at 37°C remained desensitized when a new dose of fMLF was added (shown in the inset). The curves shown are from a representative experiment. Abscissa, time of study after the addition of the second dose of fMLF (min), but in addition, the time from transfer of the samples from 15°C to 37°C is shown in parentheses; ordinate, superoxide production (arbitrary luminescence units).

at 15°C in the presence of galectin-3 also did not respond with a respiratory burst when transferred to 37°C, and no response was obtained when the cells were triggered with a new dose of fMLF, immediately after warming (within 5 min) i.e., the cells were desensitized to this agonist (not shown). However, in contrast to the cells preincubated with fMLF alone that were still desensitized after 1 h, neutrophils that were interacting with fMLF and galectin-3 in combination reemerged from the desensitized state after 20 min at 37°C and were thus responsive to a secondary fMLF stimulation (Figure 2).

Neutrophils that were interacting with fMLF and galectin-3 in combination reemerged from the desensitized state after a certain time at 37°C, and this effect was dependent on the carbohydrate-binding activity of the galectin-3 as illustrated by the facts that lactose, a competitive inhibitor, reversed the effect (Figure 3) and that the galectin-3 R186S mutant, lacking LacNAc binding capacity (Cumpste et al. 2007; Cederfur et al. 2008), had no effect in the system (Figure 3).

The inactivation of chemotactic peptides was augmented by galectin-3

One possible mechanism for breaking the desensitized state could be the inactivation of the chemotactic peptide. In order to focus on this mechanism, another experimental system was employed (see Figure 1B). In this system, the chemoattractant

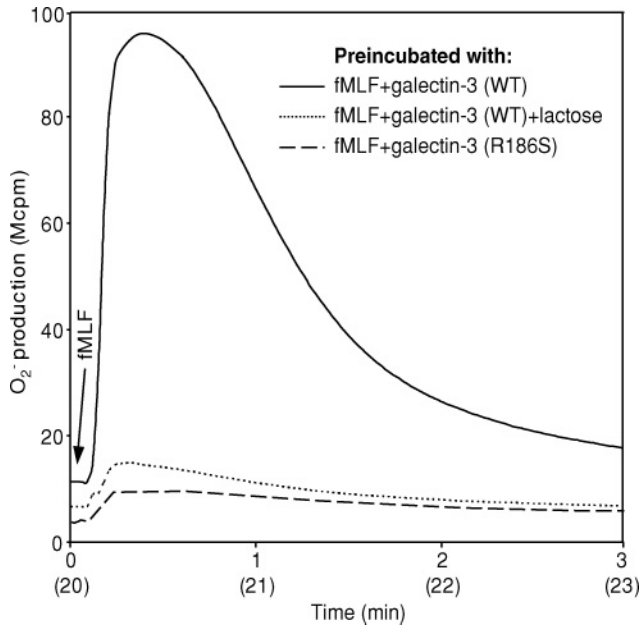


Fig. 3. Role of the lectin activity for the galectin-3 effect on neutrophil desensitization. Neutrophils ($10^6/\text{mL}$) were incubated at 15°C for 10 min together with fMLF (10^{-7} M) and galectin-3 ($20 \mu\text{g}/\text{mL}$) in the presence or absence of lactose (25 mM). The samples also contained isoluminol and HRP to allow a measurement of produced radicals (as described in Figure 1A) and in one measuring vial, the lectin was replaced by a galectin-3 mutant (R186S) that does not bind LacNAc. The samples were transferred to 37°C and after a 20 min incubation period, a new dose of fMLF was added, and the release of superoxide determined. The curves shown are from a representative experiment. Abscissa, time of study after the addition of the second dose of fMLF (min), but in addition, the time from transfer of the samples from 15°C to 37°C is shown in parentheses; ordinate, superoxide production (arbitrary luminescence units).

was added to neutrophils kept at 15°C and the mixture was then transferred to 37°C , and after different periods of time the cells were removed by centrifugation. The remaining supernatant was used to activate a new (nondesensitized) cell population. The inactivation of peptides by the neutrophils was quantified as the disappearance of the activation potential in the cell-free supernatant. The reduction in neutrophil-activating capacity in the supernatant was evident after 10–20 min of the neutrophil/fMLF mixture (Figure 4A). The activity was gradually lost, and no activation potential remained after around 60 min (Figure 4B). The same results were obtained with two other chemotactic peptides, WKYMVM and WKYMVm (Fu et al. 2006), when these replaced fMLF as the target peptide (data not shown).

The addition of galectin-3 enhanced the rate by which the neutrophil-activating capacity of the cell-free supernatant disappeared, and the galectin-3 induced augmentation was inhibited by lactose (Figure 5A). The rate of disappearance was also dependent on the concentration of added galectin-3 (Figure 5B).

The oxidative inactivation of chemoattractants has previously been described (Clark 1982) and this might be a plausible mechanism to explain the augmenting effect of galectin-3, an effect that was inhibited by lactose (Figure 5A).

The fact that the reduction of the neutrophil-activating capacity of the cell-free supernatants was associated with the disappearance of the nonoxidized form of fMLF from the supernatants and an appearance of a larger peptide corresponding in

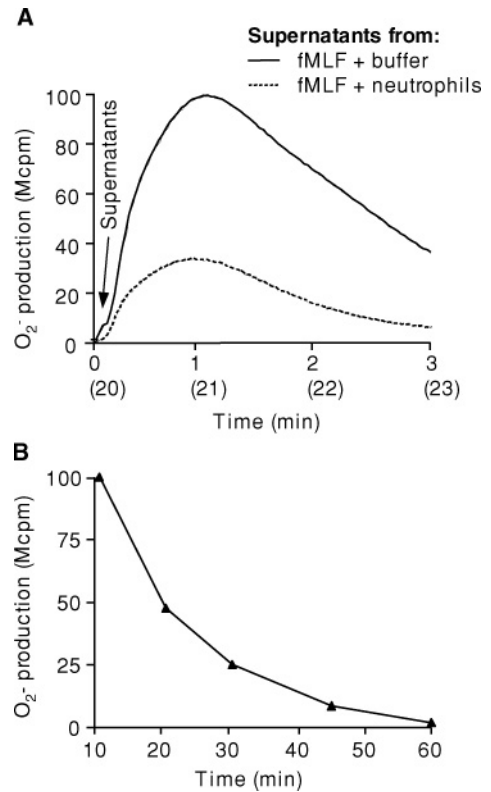


Fig. 4. Neutrophil inactivation of the chemoattractant fMLF. Neutrophils ($2 \times 10^6/\text{mL}$) were incubated together with fMLF (10^{-6} M) and the inactivation of peptides was quantified as the disappearance of the activation potential of the cell-free supernatant as described in Figure 1B. The reduction in neutrophil-activating capacity of supernatants was gradually lost. Panel A shows one representative experiment. Panel B shows the reduction in neutrophil-activating capacity with time expressed in percent of that at time 0, calculated from the peak values of the responses. Panel C shows one representative experiment with different concentrations of galectin-3. (A) Abscissa, time of study after the addition of the supernatant (min), but in addition, the time from transfer of the samples from 15°C to 37°C is shown in parentheses; ordinate, superoxide production (arbitrary luminescence units). (B) Abscissa, incubation time at 37°C before preparation of supernatant, which is used to activate a new sample of cells; ordinate, peak values of superoxide production (arbitrary luminescence units) induced by supernatants prepared after different lengths of time.

size to the oxidized form (Figure 6) support oxidative modification as the mechanism for the inactivation of the peptide.

Role of ROS for the inactivation of fMLF

A possible mechanism of the galectin-3-induced augmentation of fMLF degradation would be the increased production of ROS generated through the activation of the NADPH-oxidase. Even if, as mentioned above, preincubation of neutrophils at 15°C with fMLF±galectin-3 prohibited the proper oxidative burst to occur when increasing the temperature to 37°C (Figure 7), there is a “background” release of superoxide anions. This “background” release was somewhat increased in the galectin-3 + fMLF-treated cells compared to untreated cells and cells exposed to only one of the agonists (Figure 7). The levels of ROS production were very low, compared to the levels obtained as a result of a direct activation of nondesensitized cells with the chemoattractant fMLF, shown for comparison in Figure 7

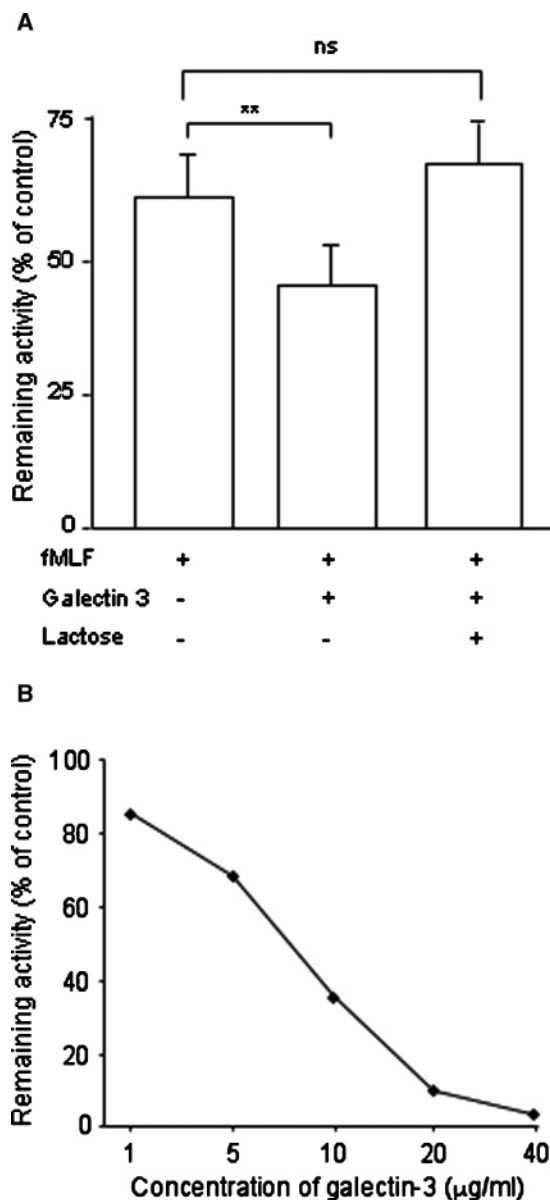


Fig. 5. Galectin-3 augments the neutrophil inactivation of fMLF. Neutrophils were incubated together with fMLF (10^{-6} M) \pm galectin-3 (20 μ g/mL) and the inactivation of peptide was quantified as the disappearance of the activation potential of the cell-free supernatant as described in Figure 1B. (A) The inactivation of fMLF was increased by galectin-3 and the increase was blocked by lactose (25 mM). The bars represent mean values \pm S.D. of three independent observations. (B) Abscissa, the concentration of galectin-3 during the preparation of supernatant, which is used to activate a new sample of cells; ordinate, peak values of superoxide production (arbitrary luminescence units) induced by supernatants prepared with different concentrations of galectin-3.

(inset) and appear as an increased background rather than a proper “burst” (Figure 7). Still, this increase in background levels of radical production could be responsible for the enhanced peptide inactivation.

Such low levels of ROS production (increased background levels rather than a burst) have previously been shown to be of biological importance (Hultqvist et al. 2006; Thoren et al. 2006, 2007) and we therefore decided to experimentally test whether

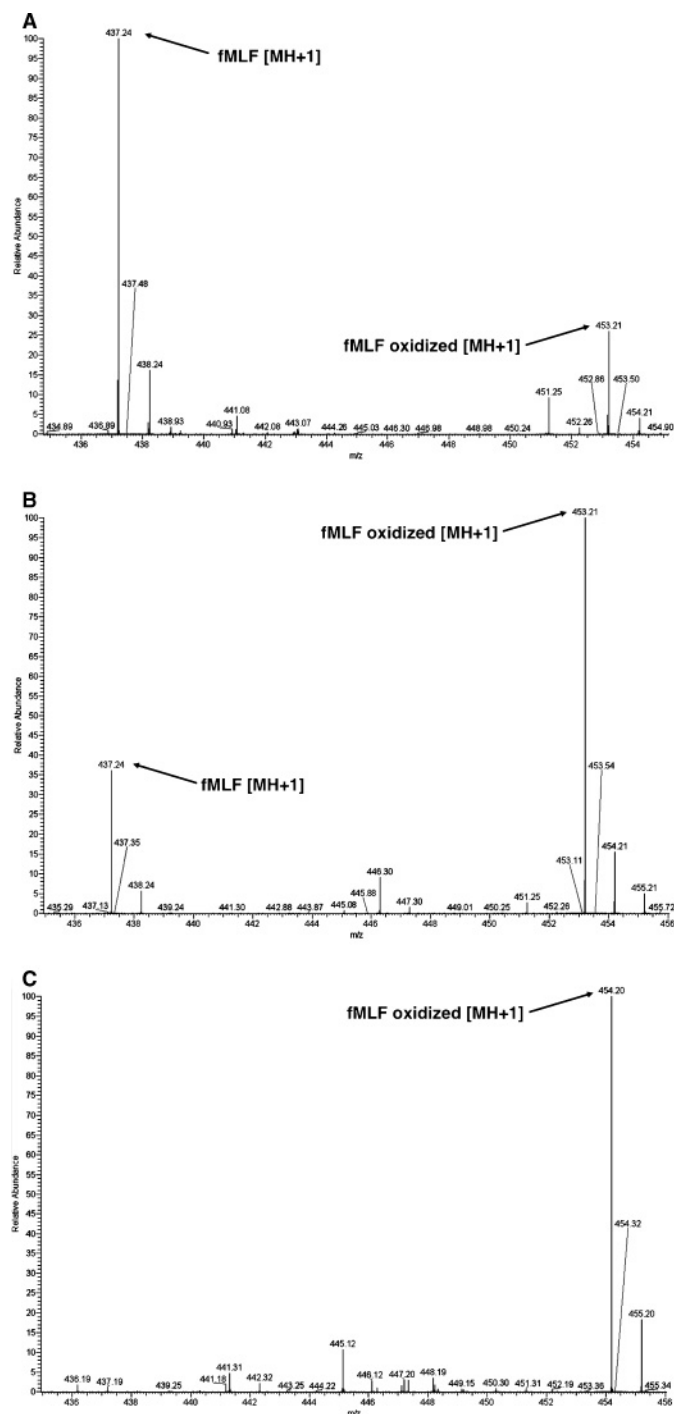


Fig. 6. Neutrophil/galectin-3-mediated oxidation of fMLF determined by static ESI-MS (LTQ-FT, Thermo Finnigan). Peptides were extracted from the neutrophil/fMLF/Galectin-3 supernatants and directly analyzed. Peptide mass measurements were based on the monoisotopic mass of the peptide 438 and 454 Da for the native and oxidized form of fMLF, respectively. The spectra shown are from (A) a control sample, (B) a sample incubated with galectin-3 (1 μ g/mL) for 20 min at 37°C, and (C) a sample incubated with galectin-3 (20 μ g/mL) for 30 min at 37°C and a comparison of the spectra shows an increase of the oxidized form at the expense of the nonoxidized form during incubation with galectin-3. The Da massshift (samples A and B) is most likely due to loss of hydrogen at one of the terminal sides of the peptide.

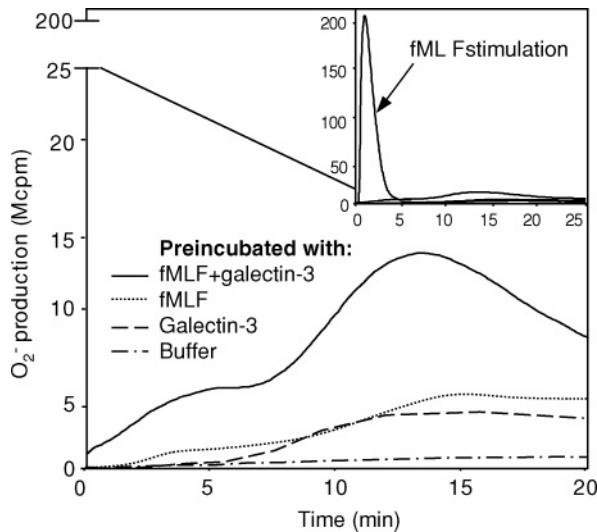


Fig. 7. Neutrophil production of superoxide anions prior to the addition of a second dose of fMLF. Neutrophils ($10^6/\text{mL}$) were incubated at 15°C for 10 min in the presence of either fMLF (10^{-7} M), galectin-3 ($20\text{ }\mu\text{g}/\text{mL}$) or in the presence of the two stimuli together. Samples also contained isoluminol and HRP to allow a measurement of produced radicals. The samples were transferred to 37°C and radical production was determined. For comparison a normal oxidative burst of fresh cells in response to fMLF is shown in the inset. The curves obtained with neutrophils incubated with fMLF, galectin-3 or both are also shown in the inset, but with another scaling of the ordinate. The curves shown are from a representative experiment. Abscissa, time of study after transfer of the samples to 37°C (min); ordinate, superoxide production (arbitrary luminescence units).

ROS production was indeed the basis for fMLF inactivation. Neutrophil incubation together with the fMLF \pm galectin-3 was performed in the presence or absence of catalase, an enzyme that consumes hydrogen peroxide. The remaining cell-free supernatants were used to activate a fresh (nondesensitized) cell population in a superoxide anion detecting assay system in which catalase has no inhibitory effect (Dahlgren and Karlsson 1999). The basal level as well as the galectin-3-enhanced peptide inactivation was totally inhibited by catalase (Figure 8A), indicating that H_2O_2 indeed played a major role in this process. In addition, the fMLF inactivation (both in the presence and absence of galectin-3) was abrogated also by the MPO-inhibiting chemical azide. Inline with this observation, no fMLF inactivation was obtained when neutrophils from an MPO-deficient blood donor was used (Figure 8B), showing that the inactivation was dependent on this enzyme. The same results were obtained when the chemotactic peptides WKYMVM and WKYVM (Fu et al. 2006) replaced fMLF as the target for the ROS (data not shown). Hence, ROS together with MPO was responsible for the peptide inactivation, and the increase in background radical production induced by coincubation with galectin-3 could fully explain the augmentation of inactivation seen in the presence of the lectin.

Discussion

The general function for galectin-3 as an inflammatory mediator/modulator has earlier been manifested through the effects on recruitment of inflammatory cells, binding of leuko-

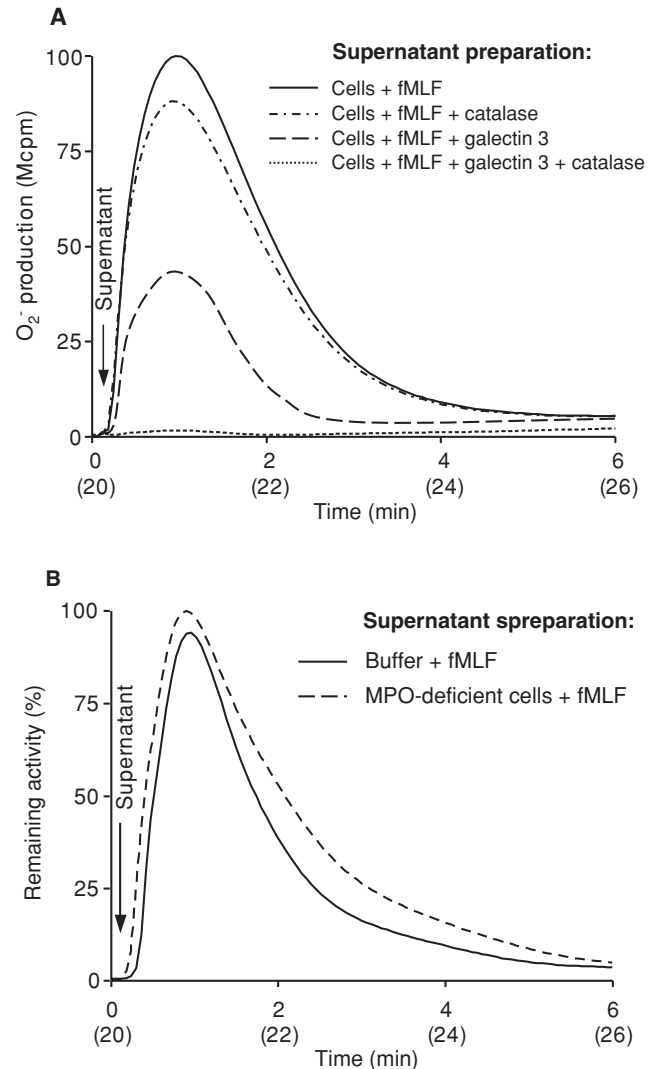


Fig. 8. Neutrophil inactivation of fMLF is dependent on ROS and MPO. Neutrophils were incubated together with fMLF (10^{-6} M) \pm galectin-3 ($20\text{ }\mu\text{g}/\text{mL}$) and the inactivation of peptide was quantified as the disappearance of the activation potential of the cell-free supernatant as described in Figure 1B. The presence of catalase ($200\text{ U}/\text{mL}$) reversed the inactivation (panel A) and no inactivation occurred when neutrophils from an MPO-deficient donor were used for preparation of supernatants (panel B). Abscissa, time of study after the addition of the supernatant (min), but in addition, the time from transfer of the samples from 15°C to 37°C is shown in parentheses; ordinate, superoxide production (arbitrary luminescence units).

cytes to endothelial cells and extracellular matrix proteins, and elimination of invading microorganisms (Liu and Hsu 2007; Farnworth et al. 2008). Here, a novel function for galectin-3 is described, the augmentation of chemoattractant inactivation, which is inline with the suggestion that galectin-3 is an important mediator of inflammation.

It has been demonstrated earlier that the chemoattractant fMLF can trigger its own inactivation when interacting with neutrophils and that methionine oxidation by the MPO-hydrogen peroxide system was the basis for loss of biological activity (Clark 1982). The fact that we could show that both the basal and the augmented inactivation of chemoattractants were inhibited by the removal of hydrogen peroxide (through the addition

of catalase) and MPO (through the addition of the inhibitor azide to control cells or through the use of peroxidase deficient cells) suggests that also the augmented inactivation process rely on the MPO-hydrogen peroxide system. This galectin-3-dependent feedback mechanism brings the concepts of oxidative activation and oxidative modulation in focus and raises questions about the in situ interplay between inflammatory cells and substances and the pathophysiological significance of oxygen radicals. With respect to the respiratory burst induced by galectin-3, we have shown that the interaction is inhibited by lactose, suggesting that the lectin interacts via terminal *N*-acetylglucosamine (LacNAc) residues present in the glycan parts of the triggering protein. There are a number of structurally and functionally diverse receptors for galectin-3 (Dumic et al. 2006), and even though the signaling receptor for galectin-3 in neutrophils is not known, we and other have shown that galectin-3 binding to LacNAc exposing membrane receptors can trigger signal transduction cascades which in turn induce numerous biochemical reactions that mediate cell activation or down regulation of particular cell functions (Almkvist and Karlsson 2004; Dumic et al. 2006). The molecular mechanisms for galectin-3-induced triggering could involve conformational changes of the glycoprotein mediated by binding of the lectin to LacNAc residues. We have earlier shown that a prerequisite for galectin induction of a respiratory burst through an activation of the NADPH-oxidase is mobilization not only of secretory vesicles but also of the proper (classical) granules. This results in increased exposure of cell surface receptors (including the suggested galectin-3 receptor; CD66) stored in the gelatinase as well as specific granules (Almkvist et al. 2001, 2002; Karlsson et al. 1998). We have also shown earlier that the peptide fMLF, an agonist for one of the members of the formyl peptide receptor family (Fu et al. 2006), works as a secretagogue that induces mobilization of galectin-3 receptors (Karlsson et al. 1998), and exposure of these receptors is possibly a prerequisite for galectin-3 activation and augmentation of peptide inactivation and augmentation of peptide inactivation.

The dose response of the chemoattractant inactivation by galectin-3 showed >90% effect at 20 $\mu\text{g/mL}$ (corresponding to $\sim 0.8 \mu\text{M}$) and 50% at less than half of that concentration. This is similar to the dose response for a number of other effects on neutrophil human leukocytes (Farnworth et al. 2008) as well as for formation of cell surface galectin-3 lattices (Nieminen et al. 2007). These concentrations are regarded as physiologically relevant although it is difficult to estimate the galectin-3 concentration at the site of action in vivo. Nevertheless, cells expressing high amounts of galectin-3 may contain $\sim 5 \mu\text{M}$ (Lindstedt et al. 1993), which would be more than enough to achieve a concentration of the lectin at the μM level, locally at the site of secretion.

The generation of ROS by the phagocyte NADPH-oxidase, is a key element of the phagocyte weaponry against pathogens, illustrated by the increased susceptibility for microbial infections associated with chronic granulomatous disease (CGD), a hereditary human disease in which the phagocytes are unable to respond with the characteristic respiratory burst when challenged with an activating agonist (Segal 2006). From a functional point of view, the term "respiratory burst" may be a misnomer, not only because the increased oxygen consumption is due to a conversion of molecular oxygen into superoxide anion rather than to elevated respiration but also due to the fact that a regular burst is not necessarily required for biological activity. A grow-

ing body of evidence implies that oxygen radicals generated by the NADPH-oxidase have regulatory functions in immunity as well as autoimmunity without any burst in activity, e.g., the low level of the production of oxidants ("background" production without any obvious burst in activity) mediates suppression of lymphocyte functions of importance for elimination of tumor cells and auto reactive cell clones (Hultqvist et al. 2006; Thoren et al. 2006, 2007; Bylund et al. 2007; Gelderman et al. 2007; Mossberg et al. 2007). The regulatory potential of an increased background level of ROS production is evident also in this study. Neutrophils interacting with galectin-3 and fMLF in combination failed to respond with a proper oxidative burst when the cells were transferred to 37°C , but the significantly increased background levels of ROS production were directly responsible for increased chemoattractant inactivation.

We used two different experimental setups to determine the inactivation of the added chemoattractants and it is obvious that when using the system with a cell-free supernatant as a source for triggering of a new cell population, the rate of inactivation was higher than when inactivation was determined as a breaking of the desensitized state. This may be dependent on a combined effect of a consumption of ROS by the oxygen radical measuring system (isoluminol/HRP) and that the desensitized state may remain for some time also after the inactivation of the chemoattractant.

The increased inactivation observed in this study relies on the lectin activity of galectin-3 and the activity is blocked by azide or catalase. Thus, the occupied galectin-3 receptors trigger neutrophils to secrete ROS that together with MPO destroy the biological activity of the chemotactic peptides fMLF, WKYMVM, and WKYMVM. Soluble agents sensitive to MPO-catalyzed inactivation are not restricted to chemotactic factors, but also protease inhibitors and bacterial toxins may be inactivated. The oxidation of functionally active methionine has been suggested to be the mechanism of loss of biological activity also for the latter compounds (Reumaux et al. 2006; Hsieh et al. 2007). Our study establishes that galectin-3 augments the neutrophil inactivation of chemoattractants through triggering the oxygen radical producing NADPH-oxidase. The feedback system of the MPO-hydrogen peroxide system puts the role of ROS production in modulation of innate immune reactions and inflammatory processes in focus. It also suggests that galectin-3 employs this negative feedback mechanism to execute a significant role as a modulator of inflammatory processes.

Materials and methods

Reagents

Galectin-3 was produced recombinantly in *Escherichia coli* and purified as described previously (Massa et al. 1993). Lectins were stored at 4°C in phosphate-buffered saline (PBS; pH 7.2) containing lactose (150 mM). When used, the lectin preparations were applied to a gel filtration column (PD10; Pharmacia, Uppsala, Sweden) in order to remove lactose and diluted to 400 $\mu\text{g/mL}$ in Krebs-Ringer phosphate buffer containing glucose (10 mM), Ca^{2+} (1 mM), and Mg^{2+} (1.5 mM) (KRG, pH 7.3). A mutant of galectin-3, in which Arg186 was replaced by a serine, was produced as described earlier (Cumpstey et al. 2007). The mutant retains affinity for lactose (about 4-fold lower) but has lost affinity for LacNAc (Cumpstey et al. 2007).

and thereby *N*-glycans in glycoproteins (Cederfur et al. 2008). The chemoattractant fMLF and isoluminol were purchased from Sigma Chemicals (St Louis, MO) and the HRP from Roche Diagnostics (Bromma, Sweden). The chemoattractants WKYMVM and WKYVM (Christophe et al. 2001) were synthesized and purified by HPLC by Alta Bioscience (University of Birmingham, Birmingham, UK).

Isolation of neutrophils

Blood neutrophils were isolated as described by Böyum et al. (1991) from buffy coats obtained from healthy volunteers, using dextran sedimentation and Ficoll–Paque (Pharmacia) gradient centrifugation. The cells were resuspended in KRG and stored on ice until use. The study was approved by the local Ethics Committee at the Medical Faculty, Göteborg University and informed consent of the donors was obtained.

Measurement of superoxide anion production

The production of superoxide anion by the neutrophil NADPH-oxidase was measured by isoluminol-amplified chemiluminescence in a six-channel Biolumat LB 9505 (Berthold Co, Wildblad, Germany) as described earlier (Lundqvist and Dahlgren 1996; Dahlgren and Karlsson 1999). In short 10^5 – 10^6 /mL neutrophils were mixed (in a total volume of 900 μ L) with horse-radish peroxidase (HRP, 4 U) and isoluminol (6×10^{-5} M) in KRG, preincubated at 37°C after which the stimulus (100 μ L) was added. The light emission was recorded continuously.

Protocols for desensitization and inactivation of chemoattractants

Neutrophils were desensitized using an earlier described protocol (Lundqvist et al. 1994; Liu et al. 1998). In short, the chemoattractant (final concentration 10^{-7} M) was added to neutrophils (10^6 /mL) kept at 15°C alone or together with galectin-3 (20 μ g/mL). After a desensitization period of 10 min the cells were transferred to 37°C and incubated for different time periods before superoxide production was measured following the addition of a second dose of the chemoattractant (Figure 1A).

In order to investigate whether the chemoattractant (fMLF, WKYMVM, and WKYVM were tested) had been inactivated, neutrophils (2×10^6 /mL) were incubated with the chemoattractant (10^{-6} M) for different periods of time, after which the cells were removed by centrifugation and the remaining supernatant was used to activate a fresh neutrophil population (Figure 1B). The inactivation of the peptides was quantified as the disappearance of activation potential in the cell-free supernatant. A brief description of the two different experimental protocols is given in Figure 1.

Modification of fMLF was also determined by static ESI-MS (LTQ-FT, ThermoFisher Scientific, Germany). Peptides were extracted from the neutrophil/fMLF/Galectin-3 supernatants by using C18 ziptips according to the manufacturers (Millipore) instructions and directly analyzed; 100 spectra per sample were collected in the mass range 400–500 *m/z* and the relative abundance between the native and the oxidized form of the peptide was determined through the intensity of the peaks. Peptide mass measurements were based on the mono isotopic mass of the protonated peptide, 438 and 454 Da, for the native and oxidized form of fMLF, respectively. The mass spectrometry analysis

was performed at the Proteomics Core Facility, University of Göteborg, Sweden.

Funding

The King Gustaf V 80-Year foundation (to C.D., A.K., and J.B.), the Swedish Society for Medicine (to H.F.), the Swedish Medical Research Council (to H.F., J.B., A.K., and C.D.), the Swedish Rheumatism Association (to A.K.), the Swedish Foundation for Strategic Research Network of Inflammation Research (to H.L., A.K., C.D.), and the Swedish state under the ALF-agreement (to H.L., J.B., A.K., and C.D.).

Conflict of interest statement

None declared

Abbreviations

CGD, chronic granulomatous disease; CRD, carbohydrate-recognition domain; fMLF, formylmethionyl-leucyl-phenylalanine; FPR1, formyl peptide receptor; GPCR, G-protein coupled receptor; MPO, myeloperoxidase; ROS, reactive oxygen species.

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