Characterization of two ELISAs for NGAL, a newly described lipocalin in human neutrophils

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Abstract

NGAL is a newly described member of the lipocalin protein family, secreted from specific granules of human neutrophils upon activation of the cells. Its ability to bind the bacterial chemotactic formylpeptide FMLP indicates that NGAL may have modulatory effects on the immune response. We here describe monoclonal and polyclonal antibodies against NGAL, which can be used for Western blotting and immunohistochemistry, and furthermore describe two ELISAs using either exclusively the polyclonal anti-NGAL antibodies or the polyclonal and monoclonal antibodies in combination. The assays are equally specific, reproducible, accurate, and sensitive, with a detection limit of 32 ng/l. The antibodies and assays will be valuable tools in the future investigation of NGAL expression in inflammatory and malignant disorders and in the elucidation of the function of NGAL as a modulator of the inflammatory response.

Keywords: Lipocalin; Monoclonal antibody; Neutrophil gelatinase associated lipocalin; Specific granule; Subcellular localization

1. Introduction

NGAL, neutrophil gelatinase associated lipocalin, a new member of the lipocalin family, was recently isolated from human neutrophils (Kjeldsen et al., 1993; Xu et al., 1994a). In spite of a limited degree of overall sequence similarity, the lipocalins share certain highly conserved motifs that are responsible for their common tertiary structure with an eight-stranded anti-parallel beta-barrel surrounding a hydrophobic core (Cowan et al., 1990; Flower et al., 1991). The lipocalins now encompass more than 25 members (including proteins like retinol binding protein, $\alpha_1$-microglobulin, $\beta$-lactoglobulin, apolipoprotein D, odorant binding protein) and are characterized by their ability to bind small lipophilic substances in their hydrophobic core, and thereby serve as transporters of substances like retinol, biliverdins, cholesteryl esters, tastants, odorants and prosta-glandins (Kjeldsen, 1995; Schenkel et al., 1995). It has been emphasized that lipocalins seem to be able to bind several different ligands rather than being specific for one (Godovac-Zimmermann, 1988).

NGAL exists in a monomeric and a homodimeric form, in addition to being partly covalently com-
plexed with gelatinase (Triebel et al., 1992; Kjeldsen et al., 1993). Northern blotting of a variety of tissues demonstrated the presence of mRNA for NGAL only in neutrophil precursors (Bundgaard et al., 1994), where the protein is biosynthesized at the myelocyte/metamyelocyte stage and directed to specific granules, from where it is exocytosed upon activation of the mature neutrophil (Kjeldsen et al., 1994a; Borregaard et al., 1995). NGAL has been demonstrated to bind the bacterial chemotactic peptide FMLP (Allen et al., 1989; Sengelov et al., 1994) and may bind lipopolysaccharide and other lipophilic inflammatory mediators. NGAL may therefore function as a modulator of the inflammatory response by preventing further recruitment and activation of neutrophils in the inflammatory/infectious focus.

As an aid for future studies of NGAL expression and function, we here present the generation of specific polyclonal and monoclonal antibodies directed against NGAL, and describe two specific, sensitive, and reproducible ELISAs for NGAL.

2. Materials and methods

2.1. Purification of native and recombinant NGAL (rNGAL)

Native NGAL was purified from isolated neutrophils as previously described (Kjeldsen et al., 1993). Neutrophils were stimulated with phorbolmyristate-acetate and the exocytosed proteins precipitated with 18% polyethylene glycol (PEG). Non-precipitated proteins were subjected to cation exchange chromatography (MonoS), followed by gel filtration on Superose 12 (chromatography was performed on FPLC (Pharmacia, Uppsala, Sweden)).

A cDNA encoding NGAL was cloned in the pGEX-4T-3 vector (Pharmacia) and transfected in the E. coli strain XL1-blue. NGAL was thereby expressed as a fusion protein with glutathione S-transferase (from Schistosoma japonicum) with a recognition site for thrombin cleavage between the NGAL and the glutathione S-transferase parts of the fusion protein. The fusion protein was purified using glutathione-Sepharose from an XL1-blue bacterial lysate. rNGAL was eluted after cleavage with thrombin and purified to homogeneity on MonoS (for details see Bundgaard et al., 1994).

2.2. Generation and purification of polyclonal rabbit anti-rNGAL antibodies

Immunization of rabbits was carried out by Dako (Glostrup, Denmark) by repetitive injections of rNGAL in incomplete Freund’s adjuvant at an NGAL concentration of 0.2 mg/ml. The anti-NGAL antisera was affinity purified on NHS-activated Superose, to which 1.5 mg of purified native NGAL (a mixture of monomeric and dimeric NGAL) had been coupled according to the instructions given by the manufacturer. The bound antibodies were eluted from the NGAL column in 3 M KSCN in PBS, and subsequently dialysed against PBS containing 0.1% sodium azide. Aliquots of the antibodies were biotinylated essentially as described (Wilchek and Bayer, 1990).

2.3. Generation of monoclonal anti-NGAL antibodies

Each of four CF1 × BALB/c F1-hybrid mice (female, 6–8 weeks old) received per immunization 0.5 ml of vaccine, prepared by adsorption of 25 μg monomeric (two mice) or 25 μg dimeric NGAL (two mice) onto 1 mg of Al(OH)3 in PBS. Immunizations were performed intraperitoneally and repeated every 2 weeks. Mice with high antibody titers (strong signal from serum diluted 1/4000) were selected for fusion. 4 days prior to fusion, the selected mouse received 0.5 ml of vaccine intraperitoneally.

Fusions were performed essentially as described by Köhler and Milstein (Köhler and Milstein, 1975) as modified by Reading (1982) with the myeloma cell line X63 Ag 8.6.5.3. and with PEG as fusogen. Positive clones were screened in ELISA as described below. Cells from positive wells were recloned three times using the limiting dilution method (Goding, 1983).

2.4. Screening of hybridoma culture supernatants for anti-NGAL reactivity

Supernatants from hybridoma cultures were screened for anti-NGAL reactivity in four different
ELISA systems (referred to as 1–4). Plates were coated overnight with (1) purified monomeric NGAL (31.25 ng/ml), (2) purified dimeric NGAL (31.25 ng/ml), or (3) and (4) rabbit anti-NGAL diluted 200-fold (Kjeldsen et al., 1993). Coating was performed in carbonate buffer (50 mM Na₂CO₃/NaHCO₃, pH 9.6). Plates were then blocked in 200 µl/well of buffer B (0.5 M NaCl, 3 mM KCl, 8 mM Na₂HPO₄/KH₂PO₄, pH 7.2, 1% Triton X-100, 1% BSA). Blocking was followed by incubation with monomeric NGAL (62.5 ng/ml) (31, and dimeric NGAL (62.5 ng/ml) (4). In (1) and (2) NGAL will be partially denatured through binding to the plastic surface of the wells, whereas in (3) and (4) NGAL is in its ‘native’ form through its binding to the anti-NGAL antibody. All plates (1–4) were then incubated with hybridoma supernatants diluted 1/10, followed by incubation with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako) diluted 1/1000. The subsequent steps were performed as for the NGAL ELISAs described below. Three monoclonal antibodies against NGAL were identified and designated 211-1, 211-2, and 711-5 respectively. The antibodies were purified from culture supernatants on Protein-G, using the Mab Trap GII from Pharmacia.

2.5. SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed essentially as described by Laemmli (1970) and Towbin et al. (1979), respectively. Protein was transferred in 10 mM CAPS, pH 11.0, 10% methanol. Additional binding sites were blocked by incubation of the nitrocellulose blots in 3% BSA, 10% goat serum in PBS or 5% skim milk in PBS. Blots were incubated overnight with primary antibodies, followed by a 2 h incubation with peroxidase-conjugated secondary antibodies (swine anti-rabbit immunoglobulins or rabbit anti-mouse immunoglobulins, both obtained from Dako). Color was developed using the metal-enhanced DAB chromogen kit from Pierce.

2.6. NGAL ELISA

Two different ELISAs were developed for measuring NGAL. In both assays, plates were coated overnight with affinity purified rabbit anti-NGAL antibodies diluted 1/5000 (158 ng/ml) in carbonate buffer (50 mM Na₂CO₃/NaHCO₃, pH 9.6). Additional binding sites were then blocked in 200 µl/well of buffer B (0.5 M NaCl, 3 mM KCl, 8 mM Na₂HPO₄/KH₂PO₄, pH 7.2, 1% Triton X-100, 1% BSA). Samples and standard (monomeric NGAL in a concentration ranging from 31.25 to 2000 ng/l) were then applied, followed by either (1) biotinylated affinity-purified rabbit anti-NGAL diluted 1/10000 (72 ng/ml) and peroxidase-conjugated avidin (diluted 1/2000, Dako) or (2) monoclonal anti-NGAL (clone 211-1) diluted 1/400 (935 ng/ml) and peroxidase-conjugated rabbit anti-mouse immunoglobulin antibodies (diluted 1/2000, Dako). All incubations were carried out in 100 µl/well, unless otherwise stated. Antibodies and samples/standard were diluted in buffer B. Color was developed by a 30 min incubation in 0.1 M sodium phosphate/0.1 M citric acid buffer, pH 5.0, containing 0.04% o-phenylenediamine and 0.03% H₂O₂ (100 µl/well), and stopped by addition of 100 µl/well 1 M H₂SO₄. The plates were washed three times in buffer A (0.5 M NaCl, 3 mM KCl, 8 mM Na₂HPO₄/KH₂PO₄, pH 7.2, 1% Triton X-100) between all steps. Before color development an additional wash in 0.1 M sodium phosphate/0.1 M citric acid buffer was included. Absorbance was read at 492 nm in a Multiscan Plus ELISA-reader (Labsystems, Helsinki, Finland). All steps were performed at room temperature.

2.7. Subcellular fractionation

Neutrophils were isolated as previously described (Kjeldsen et al., 1993). Isolated neutrophils at 3 x 10⁷/ml were incubated for 5 min on ice in diisopropylfluorophosphate (5 mM, Aldrich Chemical Co., Milwaukee, WI), followed by centrifugation and resuspension in the initial volume of disruption buffer (100 mM KCl, 3 mM NaCl, 1 mM ATPNa₂, 3.5 mM MgCl₂, 10 mM piperoxidase-N,N’-bis[2-ethanesulfonic acid], pH 7.2) containing 0.5 mM phenylmethylsulfonyl fluoride (Sigma). Cells were disrupted by nitrogen cavitation (pressurized for 5 min) as described (Borregaard et al., 1983). Nuclei and intact cells were pelleted by centrifugation at 400 x g for 15 min. 10 ml of the postnuclear supernatant (S₁) was applied on top of a 3 x 9 ml three-layer Percoll
(Pharmacia) gradient (1.05/1.09/1.12 g/ml) containing 0.5 mM PMSF and centrifuged at 37000 × g for 30 min (Kjeldsen et al., 1994b). This resulted in a gradient with four visible bands, from the bottom designated the α-band, the β₁-band, the β₂-band, and the γ-band. The cytosol was present above the γ-band on top of the Percoll. The gradient was collected in 35 equally sized fractions by aspiration from the bottom of the tube. Fractions were assayed for NGAL by the two ELISAs described above and in addition for gelatinase, lactoferrin, myeloperoxidase and HLA (Kjeldsen et al., 1994b).

2.8. Immunohistochemistry

Immunohistochemical staining of neutrophils was performed as previously described (Borregaard et al., 1995). Cytospin preparation of isolated neutrophils were fixed in 4% formaldehyde in 0.1 M phosphate buffer pH 7.0 for 20 min at room temperature and permeabilized by incubation with 1% Triton X-100 in 50 mM Tris/HCl pH 7.6, 150 mM NaCl (TBS) at room temperature for 30 min. Unspecific binding was blocked by incubating for 10 min with TBS containing 1.0% BSA. Binding of primary antibody was performed during a 1 h incubation at room temperature with antibodies diluted in TBS with 0.25% BSA. Primary antibodies were either rabbit anti-rNGAL, monoclonal anti-NGAL (211-1, 211-2, or 211-5) or non-immune serum (Dako). The slides were then washed three times in TBS and incubated for 1 h with either alkaline phosphatase conjugated swine anti-rabbit immunoglobulin (Dako) or alkaline phosphatase conjugated rabbit anti-mouse immunoglobulin (Dako) both diluted 50-fold in TBS 0.25% BSA, washed twice in TBS, and incubated in 100 mM Tris/HCl pH 9.6 for 20 min with Fast-Red (Kem-En-Tec, Copenhagen, Denmark) as recommended by the manufacturer. After washing in running tap water, the slides were counterstained in Mayer’s hematoxylin for 3 min, washed, and mounted.

2.9. Protein measurements

Protein concentration was determined by the method described by Bradford following the instructions given by the manufacturer (BioRad). Catalase was used as standard.

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<th>MW (kDa)</th>
<th>200 &gt;</th>
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<th>69 &gt;</th>
<th>46 &gt;</th>
<th>30 &gt;</th>
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Fig. 1. Western blotting of postnuclear supernatant with polyclonal and monoclonal anti-NGAL antibodies. A postnuclear supernatant (from 7 × 10⁷ neutrophils/ml) was diluted 10-fold and electrophoresed in the absence (lanes marked ‘A’) or presence of β-mercaptoethanol (lanes marked ‘B’) on a 5–20% gradient gel (rabbit-a-rNGAL) or 12% gels (monoclonal antibodies). The gels were transblotted onto nitrocellulose membranes in 10 mM CAPS, pH 10.5, 10% methanol, blocked in 5% skim milk and incubated overnight in primary antibodies in the following concentrations: polyclonal rabbit-a-rNGAL (32 ng/ml), monoclonal 211-1 (1.9 µg/ml), monoclonal 211-2 (15 µg/ml), 211-5 (7 µg/ml). After washing in PBS with 0.5% BSA, the blots were incubated for two hours in peroxidase conjugated secondary antibodies. The blots were then developed in the metal enhanced DAB kit from Pierce.
3. Results

3.1. Antibodies

Three monoclonal anti-NGAL antibodies (211-1, 211-2, and 211-5) could be identified by screening culture supernatants from hybridomas obtained by fusion of the myeloma cell line X63 Ag 8.6.5.3. with spleen cells isolated from mice immunized with either monomeric or dimeric NGAL. Monoclonal 211-1 had the highest affinity for NGAL followed by 211-2 and 211-5 respectively. The clones 211-1 and 211-2 recognized monomeric and dimeric NGAL equally well in both denatured and 'native' forms, as tested by the ELISA described in methods, whereas denaturation of NGAL markedly diminished the reactivity of clone 211-5 (not shown).

Polyclonal antibodies were obtained by immunization of rabbits with rNGAL. These antibodies were subsequently affinity-purified. The specificity of the polyclonal and the three monoclonal antibodies was assessed by Western blotting of a postnuclear supernatant from human neutrophils, electrophoresed under non-reducing as well as reducing conditions (Fig. 1). The polyclonal antibody and the monoclonals 211-1 and 211-2 recognized all three forms of NGAL present in a postnuclear supernatant, that is the 25 kDa monomeric NGAL, the 46 kDa dimeric NGAL, and the 135 kDa NGAL/gelatinase heterodimer. Under reducing conditions only one 25 kDa band was observed, in accordance with previous findings (Kjeldsen et al., 1993). No other bands were present, indicating that all antibodies were specific for NGAL. 211-1 recognized NGAL in a lower concentration than did 211-2, indicating a higher affinity of this monoclonal antibody for NGAL. The monoclonal 211-5 recognized the NGAL/gelatinase heterodimer.

Fig. 2. Immunohistochemical staining of NGAL with polyclonal and monoclonal anti-NGAL antibodies. Immunohistochemical staining of cytospin preparations of isolated neutrophils was performed as described in methods. The following primary antibodies were used: rabbit-α-NGAL (a): monoclonal 211-1 (b); monoclonal 211-2 (c); monoclonal 211-5 (211-5 showed no staining and is not shown). Note that only neutrophils are positive, scattered eosinophils are negative. When anti-NGAL antibody was replaced by preimmune serum, no staining was observed (not shown).
complex whereas the dimeric NGAL band was very weak, while no 25 kDa band was obvious under non-reducing conditions. Under reducing conditions the reactivity was completely lost. While polyclonal antibodies usually retain their reactivity against proteins when these are denatured during SDS-PAGE and transblotting, this is not always the case for monoclonal antibodies. Our findings indicate that the epitopes recognized by 211-1 and 211-2 are conserved under denaturing and reducing conditions, while 211-5 does not recognize the unreduced, denatured forms very well and not at all after reduction. The epitopes recognized by 211-1 and 211-2 differ, since reactivity against NGAL could be accomplished in a sandwich ELISA employing these two antibodies, where 211-2 was biotinylated allowing for specific recognition by HRP-avidin complex (data not shown).

3.2. Immunohistochemistry

We now investigated, whether the monoclonal anti-NGAL antibodies could be used for immunohistochemistry. The monoclonal 211-5 did not recognize NGAL in immunohistochemical staining of paraformaldehyde fixed neutrophils (not shown), in contrast to 211-1 and 211-2 (Fig. 2). The polyclonal antibodies also recognized NGAL in fixed neutrophils as expected (Fig. 2).

3.3. ELISA

We set up two ELISAs for the quantitation of NGAL, either by exclusively using polyclonal affinity purified anti-rNGAL antibodies (with biotinylated antibody as the second layer), henceforth referred to as assay A, or by combining the affinity-purified polyclonal anti-rNGAL antibody with the monoclonal antibody 211-1, henceforth referred to as assay B (see materials and methods). In both assays, monomeric NGAL was used as standard. Standard curves for the two ELISAs are shown in Fig. 3. Both had a detection limit of approximately 32 ng/l, and demonstrated near linearity in the range from 250 to 2000 ng/l. Parallelism between the standard-curve and serial two-fold dilutions of either serum or a postnuclear supernatant from human neutrophils was observed in both assays (Fig. 4). This indicates, that irrelevant proteins present in serum or neutrophil samples are unlikely to interfere with the assay. Two other lipocalins, \(\alpha\)-microglobulin and orosomucoid are present in serum in quantities several orders of magnitudes higher than the measured NGAL content, which indicate that the assay is specific for the measurement of NGAL in serum. Intra- and inter-assay variations were determined for a serum sample and for a postnuclear supernatant from human neutrophils. Assay A had an average intra-assay variation of 4.2% (5.3% for the serum sample, 3.1% for the neutrophil sample, \(n = 18\) for both), whereas the average inter-assay variation was 11.4% (11.4% for both the serum sample (\(n = 5\)) and neutrophil sample (\(n = 9\))). Assay B had an average intra-assay variation of 6.0% (4.8% for the serum sample, 7.2% for the neutrophil sample, \(n = 18\) for both), whereas the average inter-assay variation was 12.1% (12.6% for the serum sample (\(n = 9\)) and 11.7% for the neutrophil sample (\(n = 12\))). The accuracy of the ELISAs was investigated by adding different amounts of purified NGAL to either a serum sample or a
postnuclear supernatant from human neutrophils, with subsequent measurement of the NGAL content. For assay A the average recovery of NGAL was 99.4 ± 2% (postnuclear supernatant, \( n = 4 \)) and 100.1 ± 3% (serum, \( n = 4 \)). For assay B the average recovery of NGAL was 105.1 ± 2% (postnuclear supernatant, \( n = 4 \)) and 108.5 ± 4% (serum, \( n = 4 \)).

### 3.4. NGAL complexes

As demonstrated above by Western blotting, NGAL exists in neutrophils in a monomeric, in a homo-dimeric form and as a hetero-dimeric covalent complex with gelatinase. Purified neutrophil gelatinase contains NGAL, due to this complex formation between part of the gelatinase and NGAL (Triebel et al., 1992; Kjeldsen et al., 1993). We measured the NGAL content of purified gelatinase or dimeric NGAL in the presence or absence of a reducing agent (dithiotreitol), in order to investigate how the homodimerization and the physical association of NGAL with gelatinase affect the NGAL measurement obtained by both assays. We found, that the reduction of the NGAL/gelatinase complex prior to measurement resulted in a 1.8-fold increase for assay A and 1.6-fold increase for assay B in the measured NGAL content (\( n = 5 \)), whereas reduction of dimeric NGAL resulted in a 15% decrease in the measured amount by both assays. Reduction of monomeric NGAL did not affect its subsequent quantitation in either assay. This indicates, that a given amount of NGAL in complex with gelatinase will give rise to a lower measurement than the same amount of monomeric NGAL. This fact may complicate the comparison of NGAL measurements in samples, where the relative content of the different forms may vary from one sample to another. Reduction of samples prior to measurement may circumvent this problem.

### 3.5. Subcellular fractionation

We recently published, that NGAL is localized in specific granules in human neutrophils (Kjeldsen et al., 1994a). This was demonstrated by subcellular fractionation, which revealed a colocalization of the distribution profiles of NGAL and the well established specific granule marker, lactoferrin. We now wanted to compare the distribution profiles of NGAL obtained by the two assays in subcellular fractions from a three-layer Percoll gradient, and compare these measurements to Western blotting for NGAL in the same fractions. The results are shown in Fig. 5. From the Western blotting it is observed that NGAL is located in fractions 8 through 20. While the monomeric and dimeric forms clearly peak in fractions 10 and 11, it is observed that the NGAL/gelatinase complex is more evenly distributed in fractions 10 through 16, which is in accordance with the subcellular distribution of gelatinase as measured by ELISA. The distribution profiles of NGAL as measured by the ELISAs are in agreement with the Western blots, and show colocalization of NGAL with lactoferrin as expected. This confirms the allocation of NGAL to specific granules. It is furthermore observed that the quantitation

![Fig. 4](image-url)
of NGAL does not differ from one assay to the other (the distribution profiles are superimposable), which indicate that the polyclonal and monoclonal antibodies used in the assays do not differ markedly in their affinities for the different forms of NGAL present in these subcellular fractions. This is in line with the observation that no difference was observed between assay A and assay B with regards to quantitation of NGAL in purified dimeric NGAL, whereas assay B measured only 20% higher NGAL content compared to assay A in purified gelatinase (NGAL in covalent complex with gelatinase).

Fig. 5. Subcellular localization of NGAL as determined by measurement with ELISAs and by Western blotting of subcellular fractions. Subcellular fractionation of $3 \times 10^6$ neutrophils was performed on a three-layer Percoll density gradient as described in methods. The gradient was fractionated by aspiration from the bottom of the tube, and fractions were assayed for myeloperoxidase (azurophil granule marker, peak localization in fractions 7 and 3), lactoferrin (specific granule marker), gelatinase (gelatinase granule marker), NGAL with either assay A or assay B, and HLA (plasma membrane marker, peak localization in fractions 22 and 23). Percoll was removed by ultracentrifugation from 450 ml of each fraction and the material resuspended to 200 ml PBS followed by addition of an equal volume of electrophoresis sample buffer (in the absence of a reducing agent). Fractions were electrophoresed on a 5–20% gradient gel. The gels were transblotted onto nitrocellulose membranes in 10 mM CAPS, pH 10.5, 10% methanol, blocked in 5% skim milk and incubated overnight in polyclonal rabbit-a-rNGAL (160 ng/ml). After washing in PBS with 0.5% BSA the blot was incubated in swine anti-rabbit immunoglobulin (diluted 2000-fold). Color was developed in the metal enhanced DAB kit from Pierce. No NGAL reactivity was seen in fractions 1–7 and 21–35 (not shown).
4. Discussion

This paper presents a polyclonal and three monoclonal anti-NGAL antibodies. The polyclonal and two of the monoclonal antibodies specifically recognize NGAL in Western blots and detect NGAL in immunohistochemical preparations. Additionally, we present two sandwich ELISAs for NGAL using either the polyclonal antibodies exclusively (one layer biotinylated) or the polyclonal and a monoclonal antibody in combination. The assays are equally reproducible, accurate and sensitive, with detection limits around 32 ng/l. This sensitivity is at least one order of magnitude better than that obtained by Xu et al. (1994b) and Blaser et al. (1995), who recently published a RIA and ELISA, respectively, both based on the use of polyclonal antibodies against NGAL. Our two assays do not differ from each other in their affinity for the NGAL homodimer, whereas the assay based on the monoclonal antibody measures 20% more NGAL in purified gelatinase (NGAL in covalent complex with gelatinase) compared to the 'polyclonal' assay. One drawback of both assays is, that the complexation of NGAL with gelatinase seems to result in underestimation of the NGAL content, since reduction of the covalent complex leads to a 1.6–1.8-fold increase in the NGAL measurement (which is not the case, when monomeric NGAL is reduced). This is in contrast to the findings of Blaser et al, who claimed to measure more than 90% of NGAL present in complex with gelatinase (Blaser et al., 1995). The reason for this discrepancy is not clear, but may relate to the fact that the sensitivity of their assay is considerably less than the one presented here.

Xu et al. (1994b) recently suggested that NGAL measurements in tissues may provide valuable information regarding local neutrophil involvement, since NGAL is only expressed in neutrophils. Although Northern blotting of a variety of tissues demonstrated that NGAL mRNA was only expressed in immature myeloid cells from the bone marrow (Bundgaard et al., 1994), we have recently shown that NGAL synthesis is strongly induced in colonic epithelial cells in areas of inflammation as seen in adenocarcinoma, appendicitis, and inflammatory bowel disease (Nielsen et al., 1996). Furthermore, the mouse analogue of NGAL, 24p3, was found to be induced in the mouse uterus coincident with parturition and also in macrophages exposed to lipopolysaccharide (Kasik and Rice, 1995; Meheus et al., 1993). In addition, it has been found that the rat analogue, neu related lipocalin, was overexpressed in mammary cancers induced by the neu oncogene (Stoesz and Gould, 1995). Therefore, the presence of NGAL in tissues may not be a specific marker for neutrophil involvement. The antibodies and ELISAs presented in this paper will allow us to address the possible induction of NGAL synthesis in different inflammatory and malignant diseases, and specifically to investigate the value of NGAL measurements in feces as a marker for disease activity in inflammatory bowel diseases. In addition, the ELISAs and antibodies may prove valuable in the determination of the lipophilic ligands of NGAL and thus in the elucidation of the role of NGAL as a potential modulator of the inflammatory response.

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