

Expression of Lactoferrin on Neutrophil Granulocytes from Synovial Fluid and Peripheral Blood of Patients with Rheumatoid Arthritis

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ABSTRACT. *Objective.* To analyze lactoferrin expression on synovial fluid (SF) and peripheral blood neutrophils of patients with rheumatoid arthritis (RA) and to compare it with the lactoferrin expression on neutrophils from patients with osteoarthritis (OA).

Methods. Paired samples of peripheral blood and SF were obtained from 14 patients with RA and 9 patients with OA. Lactoferrin expression was evaluated on cell surfaces by cytofluorimetric analysis utilizing both polyclonal antibodies and the monoclonal anti-lactoferrin antibody AGM 2.29. Data are presented as mean fluorescence intensity.

Results. In patients with RA, the expression of membrane lactoferrin was significantly increased on SF neutrophils in comparison with those in peripheral blood. This increase was found using both polyclonal antibodies and AGM 2.29 ($p = 0.0001$, $p = 0.0017$, respectively). In patients with OA, the difference was not significant. In addition, lactoferrin expression on SF neutrophils of patients with RA was significantly increased compared with that found on SF neutrophils of patients with OA (polyclonal antibodies, $p = 0.0015$; AGM 2.29, $p = 0.005$). In patients with RA, no correlation was found between lactoferrin expression and disease activity.

Conclusion. Our results provide evidence for an activation of neutrophil granulocytes at site of inflammation in RA and indicate that lactoferrin surface expression represents a reliable neutrophil activation marker. (J Rheumatol 2003;30:220-4)

Key Indexing Terms:

RHEUMATOID ARTHRITIS
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SYNOVIAL FLUID
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Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by infiltration of the synovium with activated T cells and macrophages. On the other hand, neutrophil granulocytes (PMN) represent the predominant cell type in the synovial fluid (SF) of affected joints¹⁻³. The pathogenic role exerted by PMN in the articular destruction in patients with RA remains to be conclusively elucidated. However, evidence has been provided that reactive oxygen species, substances largely released from activated PMN, are responsible for erosion and progressive destruction of the synovial joint⁴.

PMN activation in the SF of patients with RA may be the result of cell exposure to different stimuli, including immune complexes, rheumatoid factors, cytokines, and microbial anti-

gens. Moreover, changes of surface antigen expression that reflect cell activation have been reported in PMN from SF of patients with RA⁵.

Lactoferrin (LF) is an 80 kDa iron-binding glycoprotein predominantly found in milk, tears, saliva, and other secretions^{6,7}, but is also present in the secondary granules of PMN^{8,9}. LF exerts several biological activities, including antimicrobial activity against bacteria, fungi and viruses¹⁰⁻¹⁴, as well as immunoregulatory and antiinflammatory functions¹⁵.

We previously reported that LF is not constitutively expressed on membrane of circulating PMN from healthy controls¹⁶. However, following PMN priming with phorbol myristate acetate (PMA) or tumor necrosis factor- α (TNF- α), LF becomes accessible at the cell surface. Therefore, surface membrane expression of LF may represent a reliable marker of PMN activation.

TNF- α is a proinflammatory cytokine, mainly released from activated macrophages, that plays a pivotal pathogenic role in RA¹⁷. We have demonstrated that *in vitro* PMN stimulation with TNF- α results in an increase of LF surface expression as detected by cytofluorimetric analysis¹⁶. Therefore, it is possible that TNF- α released in the SF of inflamed joints may induce LF expression on PMN recruited at the site of inflammation. The expression of several markers of inflammatory

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activation has been evaluated on PMN from SF and peripheral blood of patients with RA and other type of arthritis, while data about LF expression are lacking.

We analyzed the relationship between LF expression on PMN in paired samples of SF and peripheral blood obtained from patients with RA, and compared the LF expression on PMN from SF and peripheral blood of RA patients with that on PMN obtained from SF and peripheral blood of patients with other articular diseases, such as osteoarthritis (OA).

MATERIALS AND METHODS

Patients and sample collection. Patients were selected for this study on the basis of availability of paired peripheral blood and SF samples. Informed consent was obtained from each patient before entry into the study.

Peripheral blood and SF samples, the latter taken from knee effusion, were obtained at the same time from 14 patients with RA (10 women and 4 men; age range 22–79 yrs, median 59.5 yrs) and 9 patients with knee OA (6 women and 3 men; age range 55–76 yrs, median 64 yrs). The diagnoses of RA and OA were based on the American College of Rheumatology criteria^{18,19}. Median disease duration was 5.7 years (range 1–27) and 11 years (range 1–20) for patients with RA and OA, respectively. Ten out of 14 RA patients (71%) were rheumatoid factor positive as assessed by latex agglutination test. All RA patients were being treated with nonsteroidal antiinflammatory drugs (NSAID); 6 of them were also taking low dose glucocorticoid and a disease modifying antirheumatic drug (DMARD) (gold salts, sulfasalazine, hydroxychloroquine). All patients with OA were treated with NSAID. No patient in either group received intraarticular corticosteroid injection for at least 3 months before study entry. In the RA group, the SF leukocyte count and SF PMN count ranged from 2960 to 37,900 (median 8980) and from 2340 to 35,250 (median 7990), respectively.

For patients with RA, disease activity was assessed by means of an arbitrary disease activity index (DAI) consisting of (1) number of tender joints (53 joints evaluated; no painful joint score = 0, 1 to 4 painful joints = 1, 5 to 8 = 2, > 8 = 3); (2) number of swollen joints (46 joints evaluated, graded as for tender joints); (3) Ritchie Articular Index (0 to 4 = 0, 5 to 10 = 1, > 10 = 2); (4) duration of morning stiffness (< 60 min = 0, 60 to 120 min = 1, > 120 min = 2); (5) grip strength (mean of 3 attempts using a hand manometer, < 30 mm Hg = 3, 30 to 60 mm Hg = 2, 60 to 90 mm Hg = 1, > 90 mm Hg = 0).

As laboratory markers of systemic inflammation, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and platelet counts were determined.

Reagents. RPMI 1640, fetal calf serum (FCS), streptomycin, penicillin, caprylic acid, ammonium sulfate, human lactoferrin (purified from human milk), Freund's incomplete adjuvant (FIA), Pristane, PMA, and fluorescein isothiocyanate (FITC) conjugated F(ab')₂ fragments of sheep antibodies to mouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

All reagents utilized throughout the experiments were uncontaminated by bacterial lipopolysaccharide (LPS), as assessed by the Limulus lysate assay (Sigma).

Production of monoclonal and polyclonal antibodies to LF. The AGM 2.29 (anti-LF Mab, IgG2b)²⁰ secreting hybridoma was maintained in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, streptomycin (50 µg/ml), and penicillin (50 U/ml). Ascites was produced by injecting about 2 × 10⁶ antibody-producing cells into Pristane primed BALB/c mice.

Polyclonal anti-LF antibodies (PaLF) were produced in BALB/c mice as described^{20,21}. Anti-LF Mab and PaLF were purified from ascitic fluid and serum, respectively, by sequential precipitation with caprylic acid and 45% ammonium sulfate²². Specificity of PaLF and AGM 2.29 binding to PMN was evaluated by means of inhibition experiment as described below. Antibodies purified from preimmune BALB/c mice serum served as control immunoglobulin.

Evaluation of LF expression on PMN. Since it has been shown that PMN iso-

lation by dextran sedimentation and centrifugation on density gradients causes an increase in the expression of antigens not detectable on PMN in whole blood samples^{16,23,24}, for the analysis of LF expression, 100 µl of freshly drawn blood and SF were immediately mixed with 400 µl of ice cold PBS containing 0.1% (w/v) NaN₃. This procedure prevents artefactual upregulation of surface LF due to PMN purification¹⁶. After centrifugation at 4°C, the cell pellets were incubated with PaLF or AGM 2.29 (20 µl). Both PaLF and AGM 2.29 were diluted in ice cold PBS/0.1% NaN₃. Following a 30 min incubation at 0–4°C, cells were washed in PBS/0.1% NaN₃ and incubated with an appropriate dilution of FITC conjugate F(ab')₂ fragments of sheep antibodies to mouse IgG. Controls were performed either without a primary antibody or by incubating cells with 20 µl of normal mouse immunoglobulins. Erythrocytes were lysed by Becton Dickinson lysis solution at a dilution of 1:10, following the manufacturer's instructions. After staining, cells were fixed with 1% paraformaldehyde in PBS and stored in the dark at 0–4°C until analyzed. Cytometric analysis of surface LF was performed on a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA, USA) using Lysis II software. Ten thousand cells were counted in each analysis. PMN were gated on the basis of their forward and side light scatter. Results are given as mean fluorescence intensity (MFI).

The gating parameters were verified utilizing anti-CD15 (granulocyte-specific) and anti-CD14 (monocyte-specific) Mab (Becton Dickinson). More than 95% of PMN analyzed were anti-CD15 positive and anti-CD14 negative.

The specificity of PaLF and AGM 2.29 binding to PMN was evaluated by inhibition experiments. PMN were primed for 45 min with PMA (1 µg/ml) to induce membrane LF expression. Cells were then incubated with PaLF or AGM 2.29, as well as with PaLF or AGM 2.29 preincubated with molar excess of soluble LF (final concentration 500 µg/ml) for 1 h. The cytometry analysis was performed as described above. This experiment showed almost complete inhibition of PaLF and AGM 2.29 binding to PMN, following preincubation with molar excess of soluble LF.

Statistical analysis. Data are expressed as median and range. The Wilcoxon signed rank test for paired data and the Mann-Whitney rank sum test for unpaired data were used as appropriate. Correlation coefficients were determined using the Spearman rank correlation test. *p* values < 0.05 were considered statistically significant.

RESULTS

Comparison between SF and blood PMN from patients with RA or OA. The LF expression was compared on PMN from paired samples of blood and SF. In patients with RA, when PaLF was used, the levels of MFI found on SF-PMN (median 20.7, range 6.7–49.9) were significantly increased in comparison with those found on blood-PMN (median 7.9, range 5.6–28.3) (*p* = 0.0001). To a different extent, the values obtained in SF-PMN were in all cases increased in comparison with blood-PMN (Figure 1). Indeed, the LF expression on SF-PMN, calculated as percentage of the MFI reached by blood-PMN, was increased from 107 to 577%, with a median increase of 248%.

In patients with OA, the expression of LF on SF-PMN (median 7.2, range 4.7–11.2) did not differ significantly from that observed on blood-PMN (median 7.56, range 4.6–10.4) (Figure 2).

The results obtained utilizing the anti-LF Mab AGM 2.29 were similar to those reached with PaLF. MFI values obtained on SF-PMN from RA patients (median 12.2, range 6.8–25.2) were significantly increased in comparison with blood-SF (median 6.98, range 5.3–8.8) (*p* = 0.0017). In OA patients, the MFI documented on SF-PMN with AGM 2.29 (median 6.8,

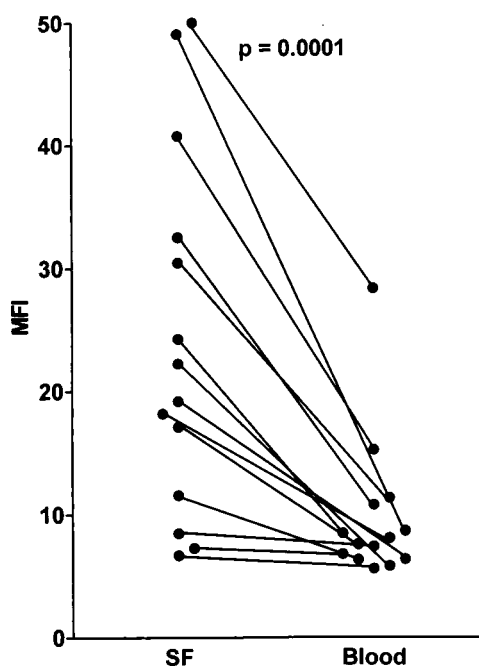


Figure 1. PMN cell surface LF expression evaluated with polyclonal anti-LF antibodies in individual paired samples of SF and peripheral blood from 14 patients with RA. MFI: mean fluorescence intensity.

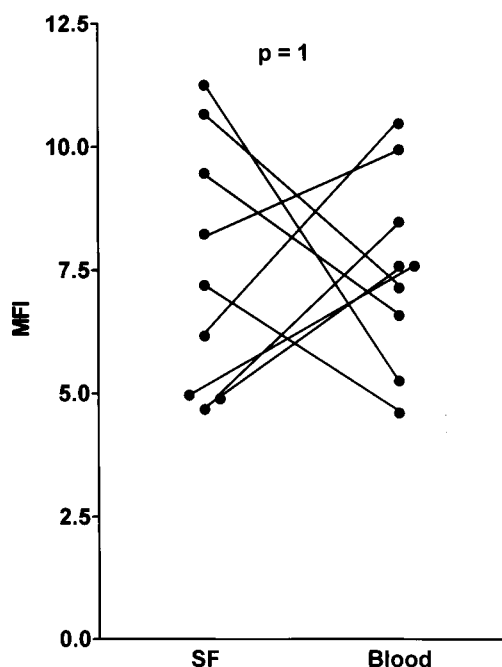


Figure 2. PMN cell surface LF expression evaluated with polyclonal anti-LF antibodies in individual paired samples of SF and peripheral blood from 9 patients with OA. Note the different scale on y axis compared with Figure 1. MFI: mean fluorescence intensity.

range 3.4–9.2) was not significantly different from that detected on blood-PMN (median 7.2, range 3–9.98) ($p = 0.3$).

Comparison between patients with RA and patients with OA.

The comparison of values obtained with PaLF on SF-PMN from patients with RA and those with OA showed the former had increased levels of LF expression ($p = 0.0015$) compared to the latter. Also utilizing AGM 2.29, a significant increase of LF expression was documented in SF-PMN of patients with RA ($p = 0.005$) in comparison to those with OA.

In contrast, no significant differences were found between LF expression on blood-PMN from RA patients and blood-PMN from patients with OA evaluated with both PaLF ($p = 0.39$) and AGM 2.29 ($p = 0.97$).

Relationship between LF expression on PMN and disease activity. In RA patients, disease activity index (DAI), evaluated at the time of sample collection, ranged from 3 to 10 (mean 5.9) and no significant correlation was found between DAI and LF expression evaluated either with PaLF ($r = 0.33$, $p = 0.25$) or AGM 2.29 ($r = -0.03$, $p = 0.9$). DAI covers only clinical manifestations. Therefore, we also evaluated the relationship between LF expression and other disease activity markers, such as ESR (median 39.5 mm, range 23–72), CRP (median 48 mg/l, range 12–192), platelet count (median 326,000/ μ l, range 197,000–510,000), and SF-PMN count (median 7990/ μ l, range 2340–35,250). No correlation was found between LF expression and ESR (PaLF: $r = 0.098$, $p = 0.73$; AGM 2.29: $r = -0.34$, $p = 0.23$), CRP (PaLF: $r = -0.01$, $p = 0.96$; AGM 2.29: $r = -0.5$, $p = 0.07$), platelet count (PaLF: $r = -0.14$, $p = 0.64$; AGM 2.29: $r = -0.52$, $p = 0.06$), and SF-PMN count (PaLF: $r = 0.19$, $p = 0.51$; AGM 2.29: $r = 0.06$, $p = 0.82$).

DISCUSSION

We evaluated by flow cytometry the LF expression on PMN in paired peripheral blood and SF samples from patients with RA and OA using both polyclonal and monoclonal anti-LF antibodies, and we found increased LF expression on SF PMN from RA patients in comparison with blood PMN. In addition, LF expression on SF PMN from RA patients was significantly higher than that found on SF PMN from OA patients. In contrast, no significant differences were found between LF expression on blood PMN from RA and OA patients. To our knowledge, this is the first report on LF expression of PMN from patients with RA.

It is noteworthy that our results were obtained on PMN incubated immediately after sample collection with ice cold buffer with the addition of sodium azide, a procedure that minimizes the possibility of artefactual PMN activation, as reported¹⁶. The possibility that LF expression found in our study was due, at least in part, to the adhesion of a soluble form cannot be formally excluded. However, if LF was simply absorbed onto PMN, a uniform LF expression on PMN should be expected, whereas a range of fluorescence intensity was always found on gated PMN, and therefore fluorescence was expressed as MFI.

PMN represent the major cellular constituent of SF in RA and their recruitment and activation in inflamed joints likely play an important pathogenic role. In this respect, upregula-

tion of several activation markers, including complement receptor 1 (CD35) and 3 (CD11b), CD 18, CD66, CD67, CD24, and CD45 has been described on the surface of SF PMN from RA patients²⁵⁻²⁸. In comparison, other PMN markers such as membrane cofactor protein (MCP, CD46) and the adhesion molecule LFA-1 (CD11a) are significantly overexpressed on peripheral blood PMN²⁷. Like other molecules such as proteinase 3 and myeloperoxidase, LF is localized in cytoplasmic PMN granules and its surface expression and release follow cell activation and degranulation²⁹. Therefore, either LF surface expression or levels of soluble LF may represent a reliable PMN activation marker.

In our study, when MFI signal of SF PMN was compared to the MFI signal of blood PMN for the individual RA patient, a MFI increase (up to 577%) was found in all instances, which provides further evidence for PMN activation at the site of inflammation. In contrast, when the same comparison was made in OA patients, in 5 out of 9 cases a major MFI signal was found on blood PMN.

Some cytokines, including interferon- γ , granulocyte-macrophage colony-stimulating factor, TNF- α , interleukin (IL) 1 α , and IL-1 β , act as PMN stimulants and induce the expression of different cell membrane receptors³⁰⁻³³. In particular, evidence has been provided that supernatants of human mononuclear cells stimulated with LPS increased CR1 and CR3 expression on mature blood PMN, this activity being exerted by TNF- α released from activated monocytes³². With respect to TNF- α effects on PMN LF expression, we have reported that priming PMN from healthy subjects with TNF- α resulted in a progressive, time-dependent increase of LF expression¹⁶, thus indicating that like other molecules, LF becomes accessible on PMN surface membrane in response to TNF- α .

It is well known that high TNF- α levels are detectable in SF of RA patients and TNF- α represents a proinflammatory cytokine mostly involved in the pathogenesis of RA¹⁷. Thus, it is possible that PMN, actively recruited at the site of inflammation from the bloodstream, express and release LF following *in situ* TNF mediated cell activation.

Besides antimicrobial activity, LF exerts different effects on immune function and inflammation. In particular, LF inhibits *in vitro* lymphocyte proliferation, as well as the production and release of proinflammatory cytokines, such as IL-1 and TNF- α from peripheral blood mononuclear cells following LPS stimulation^{34,35}. Moreover, in a murine experimental model, it has been demonstrated *in vivo* that pretreatment of LPS injected mice with LF significantly reduces IL-6 and TNF- α concentrations³⁶.

With respect to antiinflammatory activities, LF reduces the generation of extracellular hydroxyl radicals by activated PMN, by chelating iron in a noncatalytic form³⁷. In addition, LF may act as antiinflammatory molecules modulating the complement cascade activation. Indeed, LF inhibits C3 deposition on immune complexes, which is an important event for

the solubilization of immune precipitates³⁸. Therefore, although either the LF PMN expression or LF release represent a PMN activation marker, a protective function of LF at the site of inflammation could be hypothesized.

However, when in our study the rate of SF PMN LF expression was correlated with disease activity, neither positive nor negative correlation was found. Taken together, these data indicate that in RA, LF represents a PMN activation rather than a disease activity marker. The lack of correlation between SF PMN LF expression and disease activity may indicate that markers of cell activation at the site of inflammation are not always correlated with systemic disease activity. In this respect, it should be pointed out that the LF expression on blood PMN was significantly lower in comparison with SF PMN. In addition, although all patients presented with knee involvement and knee effusion, other laboratory markers of systemic inflammation, such as ESR, CRP, and platelet count, showed a wide range of values among patients.

LF may represent a possible target for autoantibodies belonging to the broad family of antineutrophil cytoplasmic antibodies (ANCA), i.e., antibodies that may be directed against different PMN cytoplasmic antigens. In a previous study we found high prevalence of ANCA in both SF and serum of patients with RA, and in some of them LF represented the target of such autoantibodies²¹. Thus, ANCA and anti-LF were detectable either at the site of inflammation or at a systemic level without difference. In this respect, it is possible that ANCA and anti-LF antibodies are not elicited as a result of increased expression of PMN activation markers at the site of inflammation. However, whatever the origin of ANCA and anti-LF in RA, we reported that their presence was not associated with more active disease²¹.

Our data show that lactoferrin, like other surface molecules, is expressed on SF PMN from patients with RA likely as a result of *in situ* PMN activation. Whether lactoferrin plays a functional role at the site of inflammation in such patients remains to be established.

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