Intraarticular Release and Accumulation of Defensins and Bactericidal/Permeability-Increasing Protein in Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Defensins and bactericidal/permeability-increasing protein (BPI) are the components of the azurophilic granules of polymorphonuclear cells (PMNC) maintaining antimicrobial protection. Both these substances have been suggested to interact with the host immune system rather than merely kill invading pathogens. We assessed concentrations of BPI and α-defensins in synovial fluid (SF) and matching blood samples of patients with rheumatoid arthritis (RA).

Methods. Matching samples of SF and blood were collected from 67 patients with RA (aged 21–73 yrs) with acute joint effusion. Blood samples from 22 healthy individuals made up a control group. Concentrations of BPI and human neutrophil peptides (HNP 1–3) were measured by ELISA. The results were related to radiological signs of destructive arthritis, duration of the disease, and laboratory markers of inflammation.

Results. BPI and HNP concentrations in SF were 10–60 times higher than in matching blood samples ($p < 0.0001$). Strong correlations between BPI and HNP concentrations were found in both blood and SF. In SF, BPI and HNP concentrations correlated to white blood cell (WBC) count ($p < 0.001$), and were associated with erosive joint disease ($p < 0.05$). In contrast, WBC count, serum C-reactive protein, or rheumatoid factor were not significantly correlated to the BPI or HNP concentrations. Serum BPI concentrations were moderately but significantly increased in RA patients compared in blood to controls ($p < 0.05$).

Conclusion. BPI and HNP are accumulated in the synovial cavity of patients with RA. Significant correlation between joint erosion and local occurrence of BPI and HNP suggests participation of these molecules in regulation of the destructive course of RA. (J Rheumatol 2003;30:1719–24)

Key Indexing Terms:
ARTHRITIS INFLAMMATION DEFENSINS

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease characterized by inflammatory cell infiltration and proliferation of synovial tissue, followed by cartilage and bone destruction. Proliferating synovial tissue is infiltrated predominantly by mononuclear cells, while polymorphonuclear cells (PMNC) are the major cell type found in acute synovial effusion of patients with RA. Degranulation of PMNC results in the release of bioactive proteases, matrix metalloproteinases, cytokines, and proinflammatory mediators into the joint cavity[1,2]. The role of the different components stored in PMN granules for the development of joint inflammation has been studied. Cytochrome b and histamine are responsible for oxidative burst, edema, and vasodilation. Prostaglandins and leukotrienes serve as chemoattractants for mononuclear cells to synovial tissues[3]. Proteases are implicated in regulation of cytokine activity and propagation of inflammation and synovial pannus formation[4], whereas matrix metalloproteinases contribute to remodeling and degradation of articular cartilage[5,6].

Besides neutral proteases and hydrolases, azurophilic granules of PMNC contain antimicrobial substances, bactericidal/permeability-increasing protein (BPI), and α-defensins. Being a part of innate immunity, these substances are rapidly delivered in response to bacterial invasion and provide a significant bactericidal effect[7,8]. BPI is a 55 kDa protein with a selective activity against Gram-negative bacteria triggered by sequestration of the outer lipid membrane. The α-defensins (human neutrophil peptides 1-3, HNP1–3) make up a group of 3 kDa peptides and constitute about 50% of the overall azurophilic granule content. These peptides *in vitro* display a broad cytolytic effect directed against bacteria, fungi, and viruses[9,10].

Both BPI and defensins have recently been suggested to interact with the host immune system, rather than merely killing the invading pathogens. BPI has been described as a

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target antigen in inflammatory bowel disease, cystic fibrosis, vasculitis, and primary sclerosing cholangitis[11]. BPI binds not only bacterial lipopolysaccharide but also endogenous phosphatidylcholine, and may participate in lipid transport[12-14]. BPI neutralizes mitogen mediated upregulation of complement receptors[15] and prevents release of tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) from monocytes[16,17], and also inhibits E-selectin expression[18]. BPI has angiostatic effects, inducing apoptosis of endothelial cells[19].

HNP not only kill invading pathogens but also display cytotoxic properties toward monocytes and certain tumors[7,10]. HNP mediate acute inflammatory responses, increasing the influx of PMNC and release of TNF-α and monocyte inflammatory protein-2 (MIP-2), thereby recruiting monocytes[20].

We report that intraarticular levels of BPI and HNP are significantly increased in patients with RA, and correlate with the destructive course of the disease.

MATERIALS AND METHODS

Patients. Samples were collected from patients with acute joint effusion who attended the rheumatology clinics of Sahlgrenska University Hospital. RA was diagnosed according to the American College of Rheumatology criteria[21]. Clinical investigations were performed in all cases and disease activity variables were recorded. At the time of synovial fluid (SF) and blood sampling all patients were receiving nonsteroidal antiinflammatory drugs. Disease modifying antirheumatic drugs (DMARD) were used by 37 patients, of which 24 used methotrexate (MTX), 3 of them in combination with cyclosporin A, and in one patient in combination with infliximab. Seven patients were taking salazopyrin, 5 were using parenteral or oral gold salt compounds, and one was taking cyclophosphamide. The remaining 24 patients had no DMARD treatment at the time of blood and SF sampling. Patients receiving monotherapy with corticosteroids were considered as having no DMARD treatment.

Recent radiographs of the hands and feet were obtained for all patients. Presence of bone erosions, defined as loss of cortical definition at the joint, was recorded in proximal interphalangeal, metacarpophalangeal, carpus, wrist and metatarsophalangeal joints. Presence of one erosion was sufficient to fulfill the requirement of an erosive disease. Presence of rheumatoid factor (RF) of any of the immunoglobulin isotypes was considered as positive.

Collection and preparation of samples. SF was obtained by arthrocentesis, aseptically aspirated, and transferred into tubes containing sodium citrate (0.129 mol/l, pH 7.4). In most cases SF was obtained from knee joints. Three samples were obtained from elbows, 6 samples were from shoulder joints. Blood samples were simultaneously obtained from the cubital vein and directly transferred into sodium citrate medium. No aspirated SF sample showed signs of bacterial/fungal infection (i.e., increased turbidity and WBC count > 50,000/ml). Blood samples from healthy individuals (n = 22, aged 22–66 yrs, mean 32 ± 7 yrs) were used in the control group. Collected blood and SF samples were centrifuged at 800 g for 15 min, aliquoted, and stored frozen at –20°C until use.

Disease activity measures. Serum concentrations of C-reactive protein (CRP) were measured with a standard nephelometric assay, with established normal range 0–5 mg. The erythrocyte sedimentation rate was measured by Westergren method, normal range 0–20 mm/h. WBC counts in blood and in SF were performed using a microcell counter (F300, Sysmex, Norderstedt, Germany). SF samples were treated with hyaluronidase before the cell count. The content of PMNC in SF was determined as a percentage of total WBC.

BPI and HNP1–3 concentrations. BPI and HNP 1–3 concentrations were determined by sandwich ELISA (HyCult, Novakemi, Enskede, Sweden) following the manufacturer's recommendations. Matched samples of plasma and SF were investigated in parallel strips.

For BPI, samples were tested in 1:10 dilutions, and for HNP 1–3 in 1:2000 dilution. The obtained values were recalculated using the reference curve.

Statistical analysis. The concentrations of BPI and HNP in blood and SF samples were expressed as mean ± SEM. Differences in matched blood and SF samples were analyzed by paired t test. Differences between groups were calculated by Mann-Whitney U test. Correlations between measures were calculated using the Z-coefficient. P values < 0.05 were considered significant.

The study was approved by the Ethics Committee of Sahlgrenska Hospital.

RESULTS

Clinical and demographic data at presentation. Matched samples of SF and blood were collected from 67 patients with RA. Patients' clinical and demographic data are presented in Table 1.

Stratification of patient data by radiological imaging showed that 44 patients had erosive joint disease; 23 patients had no erosions in recent radiographs. There was no difference in patients' ages between these 2 groups (61 ± 4 vs 62 ± 7 yrs, respectively; NS). As expected, patients in the group with erosive RA were RF-positive significantly more often (35/44 vs 12/23; chi-square 4.74, $p < 0.05$), and had longer duration of disease than patients with non-erosive RA (17 ± 2 vs 8 ± 1.6 yrs; $p = 0.015$).

Stratification of patients for disease duration yielded 20 patients with short duration of RA (< 3 yrs, mean 1.8 ± 0.3 yrs), while the remaining 47 patients had a mean disease duration of 18 ± 11 (range 5–60) years. These 2 groups were similar with respect to occurrence of RF, but differed in the frequency of erosive joint disease (8/20 vs 39/47; chi-square 12.4, $p < 0.01$).

Accumulation of BPI and HNP in SF. SF of RA patients contained high concentrations of BPI and HNP, 298 ± 74 and 3503 ± 963 ng/ml, respectively. Concentration of these proteins in RA SF was 10–60 times higher than in corresponding blood samples ($p < 0.001$ for both variables; Table

Table 1. Clinical and demographic characteristics of patients with RA.

Total no.	67 (50 women, 17 men)
Age, yrs	61 (range 25-87)
Disease duration, yrs	14 (range 1–60)
< 3 yrs (1.8 ± 0.3)	20
> 3 yrs (18 ± 11)	47
Radiographs erosive/non-erosive	44/23
RF positive/negative	47/20
Acute phase response	
CRP, mg/ml	42 (range 5–140)
ESR, mm/h	48 (range 12–98)
WBC count, x 10^9/ml	
Blood	8.1 (range 3.4–18.6)
Synovial fluid	12.2 (range 0.9–48)

Table 2. Concentrations of neutrophil defensins and BPI in RA patients and controls.

Groups	HNP 1–3, ng/ml		BPI, ng/ml	
	Blood	Synovial Fluid	Blood	Synovial Fluid
RA patients, n = 67	213 ± 9	3503 ± 963*	23 ± 5	298 ± 74*
Healthy controls, n = 22	200 ± 1	NA	18 ± 0.2	NA

* Blood versus synovial fluid, $p < 0.001$. NA: not analyzed.

2). In the control blood samples, BPI concentrations were slightly higher in patients with RA compared to controls ($p < 0.05$), while HNP levels were similar. There was no correlation between BPI or HNP levels in the matching samples of blood and SF. In contrast, concentrations of BPI and HNP correlated well to each other in both blood ($r = 0.65$, $p < 0.0001$) and SF ($r = 0.75$, $p < 0.0001$). Blood levels of BPI and HNP were not related to the acute phase reactants CRP and ESR, nor to WBC count (Figure 1a). In contrast, a significant correlation was observed between WBC count and the levels of BPI ($r = 0.52$, $p = 0.05$) and HNP ($r = 0.69$, $p < 0.0001$) in SF (Figure 1b). However, levels of BPI and HNP were not significantly related to the amount of PMNC in SF.

Correlation between concentrations of BPI and HNP and RA clinical characteristics. Comparison of the patient groups with erosive and non-erosive joint disease revealed that erosive disease was associated with significantly higher concentrations of BPI (343 ± 69 vs 206 ± 73 ng/ml; $p = 0.05$) and HNP (4374 ± 805 vs 2391 ± 946 ng/ml; $p < 0.05$) in SF compared to non-erosive disease, while blood levels of BPI and HNP in erosive versus non-erosive patients were similar (Figure 2). Since occurrence of RF might interact with the sandwich ELISA for BPI and HNP determination, we assessed relations between the presence of RF and BPI/HNP concentrations. However, neither blood nor SF levels of BPI and HNP differed in patients stratified for presence/absence of RF.

To investigate if age and duration of disease influenced the level of BPI and HNP, RA patients were stratified according to these 2 variables. Blood levels of BPI were higher in patients with short duration RA (disease duration ≤ 3 yrs) compared to those with longer disease duration (39.2 ± 11.4 vs 17.1 ± 2.8 ng/ml; $p < 0.05$). In SF, patients with short duration of RA had somewhat lower concentra-

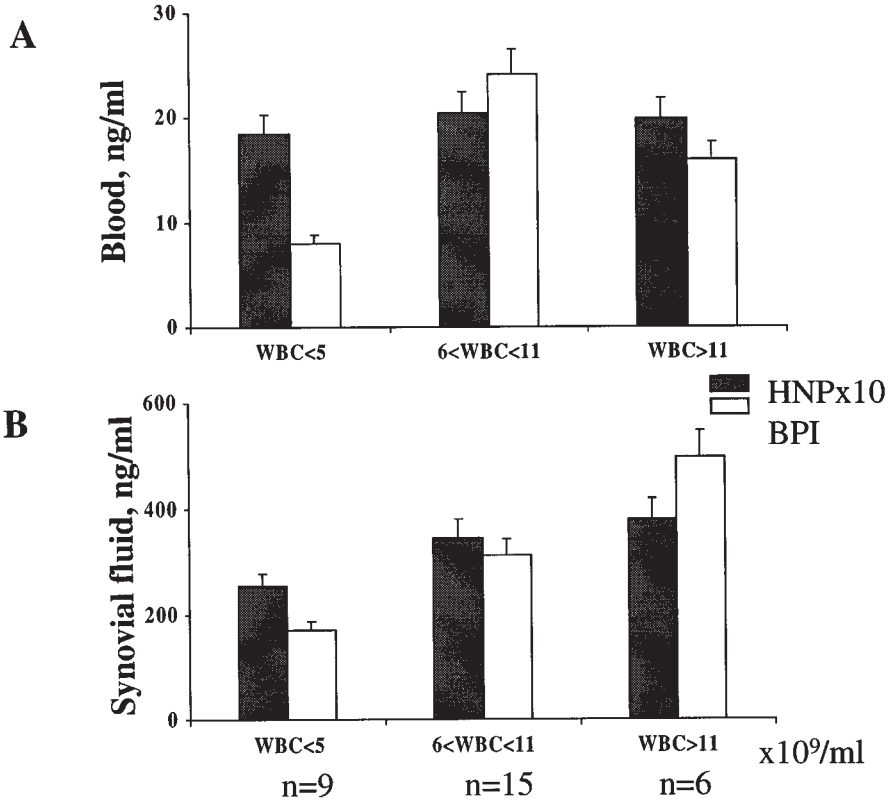

Figure 1. Relationship between WBC count and the concentrations of BPI and HNP (A) in blood; (B) in synovial fluid.

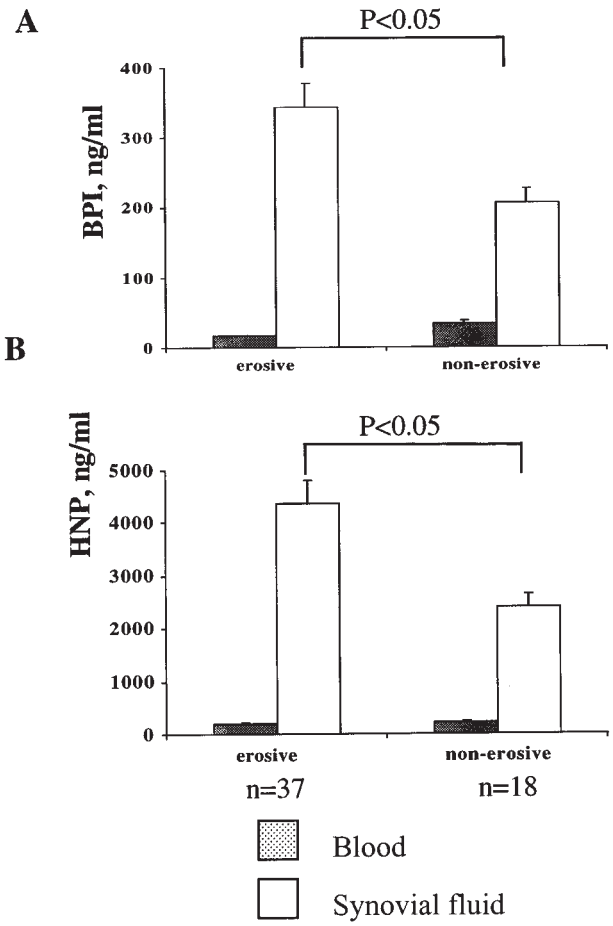

Figure 2. Accumulation of BPI and HNP in synovial fluid of patients with erosive versus non-erosive RA. Concentrations of BPI and HNP are presented as means ± SEM (ng/ml).

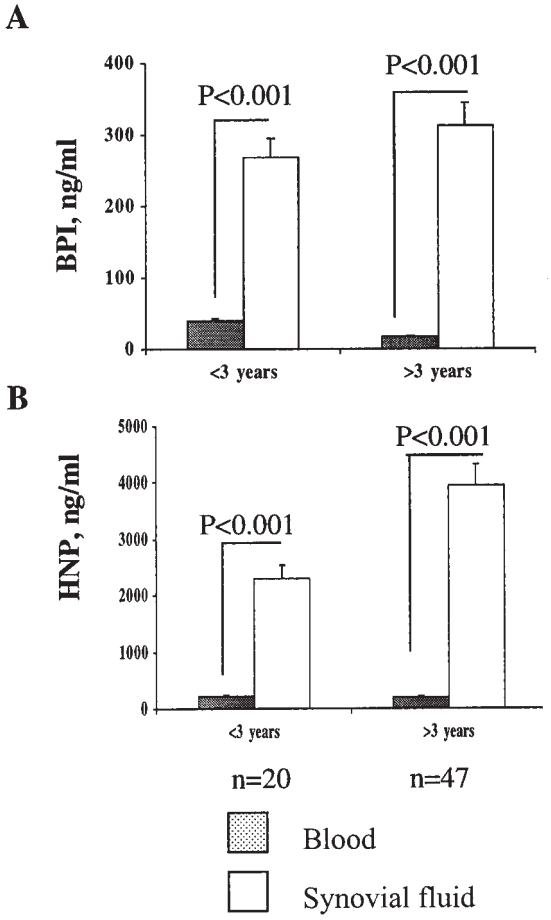

Figure 3. Influence of duration of RA on concentrations of BPI and HNP in matching blood and synovial fluid samples. Concentrations of BPI and HNP are presented as means ± SEM (ng/ml).

tions of BPI (269 ± 82 vs 313 ± 72 ng/ml; p = NS) and HNP (2310 ± 1324 vs 3941 ± 754 ng/ml; p = NS) compared to patients with disease duration > 3 years (Figure 3).

BPI concentration in SF was influenced by patients' age, and was higher in the patients older than 56 years (mean 70 ± 9 yrs, n = 47) compared to those younger than 56 years of age (mean 39 ± 8 years, n = 20) (328 ± 76 vs 178 ± 75; p = NS). Notably, older patients had significantly lower WBC count in SF compared to the younger ones (8 ± 3 vs 18 ± 6 × 10^9/ml; p = 0.027).

DISCUSSION

Our results indicate that BPI/HNP are active participants during the inflammatory process in RA. Close correlation of BPI/HNP concentrations to the destructive joint disease and an increase of BPI/HNP concentrations with duration of disease suggest their participation in the regulatory mechanisms of arthritis rather than being markers of acute inflammatory response. Significantly increased levels of BPI/HNP in the synovial cavity compared with blood strongly suggest the local production and usage of these proteins during the disease process.

We observed that the BPI/HNP concentrations in SF correlate to WBC counts, but not to PMNC counts. This finding may be interpreted in one of 3 ways: (1) an increase in BPI/HNP load in the azurophilic granules rather than increase in the total number of PMBC in the joint cavity in RA; (2) increased degranulation and apoptotic cell death of PMNC in the synovial cavity; and/or (3) production of BPI/HNP by other sources besides PMNC, e.g. monocytes/macrophage cells, B cells[22], and/or cells within the synovial tissue. All these possibilities exist in joints. The content of BPI in PMNC is known to increase with age[23,24]. This is in agreement with our finding of the highest amount of BPI in the SF of older patients. The intracellular content of HNP, in contrast, is a matter of instant regulation at different stages of cell maturation and recruitment from the bone marrow[25]. For example, HNP load is influenced by TNF-α and interferon-γ stimulation[26,27], and both these cytokines are present in inflamed RA joints. Of particular

interest in this respect are the reports of HNP synthesis by mononuclear and microvascular endothelial cells[22,28]. Both cell types are present within the joint at the very early stages of arthritis, and represent additional sources of HNP in the synovial cavity.

Both BPI and HNP mediate cytolytic activity by binding negatively charged phospholipids on the cytoplasmatic membranes of bacteria and disrupting their functional integrity[10,29]. This mechanism is not restricted to microorganisms and may be applied also to human cell membranes[12]. Indeed, BPI bound to cell-surface phosphatidylcholine molecules has been detected on monocytes and macrophages[14], and may serve as an important protection against monocyte accumulation in the joint tissues in the initial stages of RA. The receptor used by HNP to interact with mononuclear cells seems to be MHC class II. This interaction has been shown to interfere with the antigen presentation process[30]. BPI has also been found to act as a natural TNF-α inhibitor[17] and thus may facilitate protective mechanisms inside the joint cavity. Thus, both BPI and HNP seem to limit the inflammatory process in the joint. Correlation between the increased release of BPI/HNP intraarticularly and the destructive course of RA would further support this concept. On the other hand, high concentration of HNP has proinflammatory effects of its own, inducing chemotaxis of PMN, monocytes, and dendritic cells[10,31]. Neutralization of HNP by complex formation with α_2-macroglobulin, which is abundant in inflamed SF, diminishes its phlogistic properties[32,33].

Our results indicate that defensins and BPI are continuously accumulated in the inflamed joints during RA. Correlation between the concentrations of BPI/HNP and bone destruction indicates their regulatory role during the course of erosive arthritis.

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