Clinical and Experimental Rheumatology 2000; 18: 63-66.

Marked elevation of serum *N*-acetyl-β-D-hexosaminidase activity in rheumatoid rheumatoid arthritis

F. Berenbaum[1,2], L. Le Gars[1], E. Toussirot[3], A. Sanon[4], C. Bories[4], G. Kaplan[1], P.M. Loiseau[4]

[1]Department of Rheumatology, Hôpital Saint-Antoine, Paris; [2]UPRES-A 7079, Pierre and Marie Curie University - Paris VII, Paris; [3]Rheumatology Department, J. Minjoz Teaching Hospital, Besancon; [4]Biologie et Contrôle des Organismes Parasites, Faculté de Pharmacie, Université de Paris-Sud, Châtenay-Malabry, France.

Support for this study was received from Roche Laboratories.

Francis Berenbaum, MD, PhD; Laurence Le Gars, MD; Eric Toussirot, MD; Albertine Sanon, student; Christian Bories, PhD; Gérard Kaplan, MD, Professor, Department Head; Philippe M. Loiseau, PhD.

Please address correspondence and reprint requests to: Francis Berenbaum, Department of Rheumatology, Hôpital Saint-Antoine, 184 rue du faubourg Saint-Antoine, 75012 Paris, France.
E-mail: francis.berenbaum@sat.ap-hop-paris.fr

Received on April 19, 1999; accepted in revised form on September 14, 1999.

Key words: Rheumatoid arthritis, *N*-acetyl-β-D-hexosaminidase, cartilage, chondrocyte.

ABSTRACT

Objective.
To study N-acetyl-β-D-hexosaminidase (NAHase) activity in the sera of rheumatoid arthritis (RA) patients and to determine its source.

Methods.
NAHase activity in the serum and synovial fluid of RA patients was measured with p-nitrophenyl β-N-acetylglucosaminide as substrate. The p-nitrophenol released was measured spectrophotometrically in an ELISA reader. Rabbit articular chondrocytes in primary culture were stimulated with interleukin-1β (IL-1β).

Results.
Serum NAHase activity was higher in 35% of the RA patients than in healthy patients. The median activity was about twice that of the serum of healthy volunteers. RA patients with high serum NAHase activity also had more joint destruction (85%) than those with normal NAHase activity (57%, $p < 0.05$), but their inflammatory status was similar.
The source of NAHase in RA was investigated by assaying it in RA synovial fluids (SF) and measuring its release from articular chondrocytes in primary culture. NAHase activity was detected in all 23 RA SF, at a median concentration that was 2 times that of the serum. NAHase activity in the medium of articular chondrocytes was stimulated by IL-1β ($p < 0.005$ compared to unstimulated cells), suggesting that cartilage is a source of serum and SF NAHase activity.

Conclusion.
The serum concentration of the matrix hydrolase, NAHase, is higher in destructive RA than in inflammatory RA.

Introduction

Rheumatoid arthritis (RA) is a autoimmune disease whose course may vary (1). The disease results in progressive joint destruction and disability in a substantial proportion of patients (2). Being able to predict the outcome early in the disease would enable physicians to identify those patients requiring early disease-modifying therapy (3). It is therefore essential to identify biochemical abnormalities that are early indicators of outcome.

The extra-cellular matrix of cartilage is made up of proteoglycan aggregates constrained within a network of collagen fibers. Aggrecan, the major cartilage proteoglycan, consists of one or more glycosaminoglycan (GAG) chains covalently attached to a protein core by their reducing terminals. Cartilage is thus broken down by proteases (metalloproteases, acid proteases), enzymes that have been widely studied, and by glycosidases. The polysaccharide chains of GAGs are broken down by endoglycosidases, exoglycosidases and sulfatases. Hyaluronidase is a widely distributed endoglycosidase that cleaves hexosaminidic linkages. β-glucuronidase, β-galactosidases, α-L-iduronidase and *N*-acetyl-β-D-hexosaminidase are exoglycosidases. *N*-acetylglucosamine (GlcNAc) is one of the most widespread and abundant glycosaminoglycans in human cartilage (4). Terminal β-linked GlcNAc residues are released by *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.52) (NAHase), a lysosomal enzyme that hydrolyses the glycosidic linkage of β-D-*N*-acetylglucosaminides when they are present at the non-reducing end of a saccharide. The substrates for this enzyme include chondroitin sulfates, hyaluronic acid, dermatan sulfate and keratan sulfates I and II. NAHase therefore takes part in the degradation of glycoproteins, glycolipids and glycosaminoglycans (5).

This study examines the hypothesis that the degradation of cartilage in RA depends, at least in part, on enhanced NAHase activity, which can be released into the bloodstream. We assayed NAHase activity in the serum of RA patients. We then investigated the source of this enzyme by measuring NAHase activity in the synovial fluid (SF) of RA patients and by assaying NAHase in the media of articular chondrocytes cultured with and without IL-1β.

Patients and methods

Study subjects

Sixty-one healthy subjects, 34 women and 27 men, aged 18-93 years (mean 56 ± 19 years), participated in the study. No subject was on anti-inflammatory drugs and none had clinical signs or symptoms

of joint disease.
Fifty-seven consecutive patients with RA seen at the Department of Rheumatology of Saint-Antoine Hospital, who met the criteria of the American College of Rheumatology (formerly the American Rheumatism Association) (6), were studied. Patients whose disease had begun before the age of 18 years were excluded. Pregnant patients and those with infections, neoplasia, renal insufficiency, liver disturbance, or who were on cytotoxic chemotherapeutic agents were excluded. A total of 36 women and 21 men aged 23-83 years (mean 55 ± 12 years) were recruited for the study. Samples of synovial fluid from 23 active RA patients were analyzed in parallel.
X-rays were taken of the hands, feet and all joints showing clinical symptoms. The radiographs were blindly read by 2 observers and scored by the method of Larsen (7). A Larsen score > 2 for one joint or for at least one joint replacement defined the patients with joint destruction.

Clinical assessment
All joint surgery and drug treatment regimens were recorded. Of the 57 patients, 23 were on non-steroid antiinflammatory drugs (NSAIDs), 33 were receiving prednisone alone (n = 12), or prednisone plus disease-modifying antirheumatic drugs (DMARDs) (n = 17) and 4 patients were on DMARDs alone. Morning stiffness lasting longer than 30 minutes was considered positive.

Laboratory assessments
The following tests were regularly performed to evaluate disease activity: Westergren erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and serum rheumatoid factor (RF) by laser nephelometry (positivity ≥ 40 UI/ml).
p-nitrophenyl β-D-*N*-acetylglucosaminide, HAM's F12 and DMEM were from Sigma Chemical Co. (Saint-Quentin Fallavier, France). Fetal calf serum (FCS), hyaluronidase, collagenase and trypsin were purchased from Boehringer Mannheim (Meylan, France). Recombinant human IL-1β was from Immugenex (Boston, MA).
N-acetyl-β-D-hexosaminidase activity was determined with *p*-nitrophenyl β-N-acetylglucosaminide at a final concentrations of 5.83 mM. NAHase was assayed in 0.2 M sodium acetate buffer (pH 5.2). Assays (final volume, 120 µl) were carried out using 10 µl human serum diluted (1/15) for β-D-N-acetylhexosaminidase. NAHase activity in the supernatant of cultured cells were measured using 50 µl supernatant. The incubation time was 24 hr at 37°C and reactions were stopped by adding 50 µl 0.1 N NaOH / 0.8 M glycine buffer (pH 10.5). Serum and substrate blanks were included with each determination and used to correct enzyme activities. The *p*-nitrophenol formed was measured spectrophotometrically in an ELISA reader (Multiskan MS spectrophotometer) at 405 nm. One unit of enzyme activity corresponded to the liberation of 1 nmol β-D-N-acetylglucosamine/min/ml serum at pH 5.2 and 37°C for NAHase.

Isolation, culture and stimulation of chondrocytes
Chondrocytes were cultured as previously described (8). Briefly, 4-week-old rabbits were killed and the shoulders, knees and femoral heads were dissected out under sterile conditions. The articular cartilage was removed and cut into small pieces (< 0.5 mm^2) and digested at 37°C with 0.05% testicular hyaluronidase in HAM's F12 medium for 15 min, in 0.25% trypsin for 30 min, and in 0.2% clostridial collagenase for 90 min. The resulting suspension of chondrocytes was seeded onto 24-well cell culture plates (2 x 10^4 cells per well) in HAM's F12 supplemented with 10% FCS, 10 UI/ml penicillin and 100 mg/ml streptomycin. The cells were grown at 37°C in 5% CO_2, and the culture medium was changed every 3 days. The cells became confluent within 6-7 days. Only primary cell cultures were used for these studies to ensure the stability of the chondrocyte phenotype. The medium was removed from confluent cells and they were incubated in serum-free DMEM medium for 24 hrs prior to incubation with IL-1β (0.1 - 10 ng/ml) and/or TNFα (1 - 100 ng/ml) for 24 hrs.

Statistical analysis
Data were analysed using Statview software. Results are expressed as means ± SEM and confidence intervals. The Mann-Whitney U test for unpaired differences was used as appropriate. P values < 0.05 were considered to be significant.

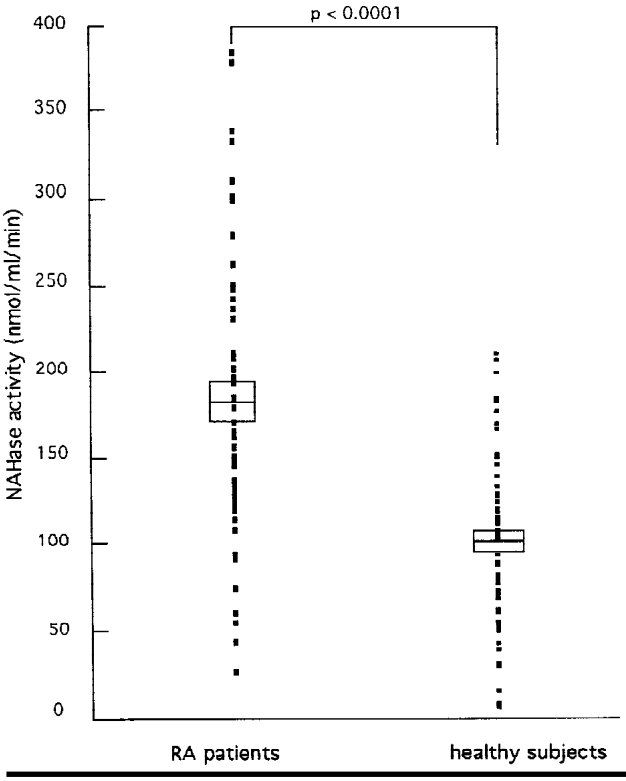

Fig. 1. Serum *N*-acetyl-β-D-hexosaminidase activity in 61 healthy subjects and 57 rheumatoid arthritis patients. Box: mean ± SEM.

Table I. NAHase activity in sera from rheumatoid arthritis (RA) patients (n = 57) and healthy subjects (n = 61).

	NAHase (nmol/ml/min)	
	RA	Controls
Mean	181.69	98.22
SEM	10.90	6.29
95% CI	159.85 - 203.54	85.64 - 110.80
	p < 0.0001	

Results

Serum NAHase activity in healthy subjects and RA patients

The NAHase activities of each of the 61 healthy subjects and 57 RA patients are shown in Figure 1. The distribution in healthy subjects is skewed and does not represent a normal distribution. The NAHase activities of men and women were not significantly different (data not shown). The mean (± SEM) NAHase activity (Table I) indicated that the NAHase activity in RA patients (181.7 ± 10.9 nmol/ml/min) was significantly higher than in healthy subjects (98.2 ± 6.3 nmol/ml/min, $p < 0.0001$), although there was an overlap between the values for normal and RA patients.

Clinical and laboratory status of RA patients according to their NAHase activity

An arbitrary cut-off was defined as the mean + 2 SD serum activity in healthy subjects. Patients with high serum NAHase activities had significantly more (i.e. 85%) destruction - as defined in the Methods section - than did those without elevated serum NAHase (57%, $p < 0.05$). In contrast the inflammatory markers, such as morning stiffness, ESR and CRP, showed no difference (Table II). This was not due to differences in age or disease duration (data not shown), and there were no differences in any of the clinical or laboratory variables of these patients. The effects of RA medication on N-acetyl-β-D-hexosaminidase activity were investigated, but no conclusions could be drawn because of the wide range of drugs being taken by the patients.

Synovial fluid-NAHase activity in RA patients

We detected NAHase activity in all 23 RA patients. The median activity was 339.7 ± 40.8 nmol/ml/min, twice the median RA serum NAHase activity (data not shown).

Table II. Comparison of rheumatoid arthritis patients (RA) according to NAHase activity.

	Normal serum NAHase activity < 199 nmol/min/ml (n = 37)	High serum NAHase activity > 199 nmol/min/ml (n = 20)	Statistical significance
Erosions	n = 21	n = 17	**p = 0.04***
Disease duration	72 ± 13	89 ± 15	NS
Morning stiffness	n = 23	n = 10	NS
Erythrocyte sedimentation rate	46 ± 5	46 ± 6	NS
C reactive protein	47.7 ± 7.7	39.0 ± 8.6	NS
Hb	12.3 ± 1.3	12.7 ± 1.2	NS
Rheumatoid factor positive	n = 20	n = 12	NS
Extra-articular disease	n = 9	n = 9	NS

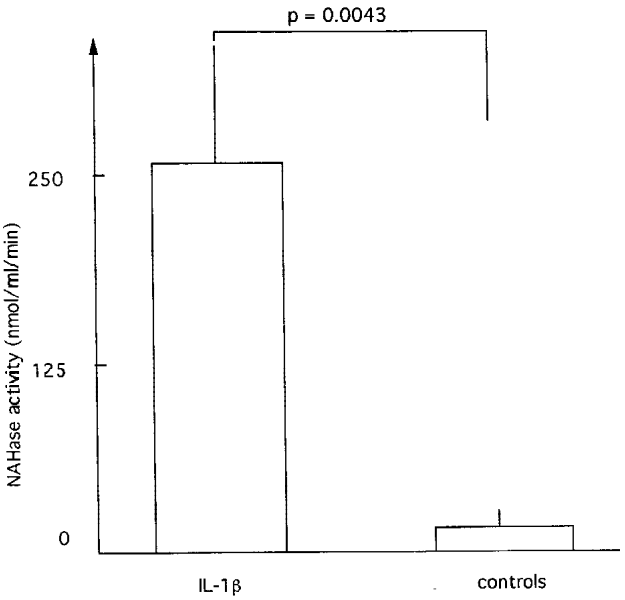

Fig. 2. *N*-acetyl-β-D-hexosaminidase activity in the medium of unstimulated and stimulated rabbit articular chondrocytes in primary culture (24 hr IL-1β, 10 ng/ml). Results are shown for 6 separate experiments.

NAHase activity in the supernatant of IL-1β-stimulated chondrocytes in primary culture

IL-1β (10 ng/ml, 24 - 48 hrs) dramatically increased NAHase activity in the supernatant of rabbit articular chondrocytes in primary culture; it was 261.83 ± 79.87 mmol/ml/min in cultures with IL-1β and 17.83 ± 10.10 nmol/ml/min ($p < 0.005$; n = 6 independent experiments) in cultures without IL-1β (Fig. 2). TNFα alone had no effect under these conditions (data not shown).

Discussion

There is compelling evidence that cartilage is broken down by enzymes that catabolize matrix components. The main enzymes involved in this catabolism are the metalloproteinases, which can be produced by synoviocytes or by chondrocytes (9). But some studies suggest that glycosidases are also involved in matrix destruction (10, 11). Our results support this, since NAHase activity was enhanced in the culture medium of articular chondrocytes grown in IL-1β, the major pro-degradative cytokine in the synovial fluid of RA patients. Based on this experimental result, we assayed NAHase activity in the serum of RA patients and healthy subjects. In agreement with a previous study (12), we found that NAHase activity is significantly elevated in RA patients. This could be due to overproduction of NAHase from the joints, since the concentration of SF-RA NAHase was twice the serum concentration. The increased release of NAHase activ-

ity into the media of activated chondrocytes in culture suggests that cartilage is a source of NAHase in RA joints, but we cannot exclude the possible participation of synovial tissue. Polymorphonuclear leukocytes are also known to contain NAHase in their lysosomes (13). Since large numbers of leukocytes are present in RA synovial fluid, these cells are likely to be another source of NAHase. The fact that RA neutrophils are more active than normal cells strengthens this hypothesis (14).

Since RA patients with high serum NAHase activity were more erosive but were not different in term of CRP levels, it is possible that NAHase is a marker of erosion that is independent of the inflammation status. However, the methods used in this study do not permit us to draw this conclusion, and a longitudinal study with discriminant analysis is needed.

Our data suggest that the activity of NAHase, a glycosidase involved in matrix degradation, is elevated in RA patients. We postulate that the source of at least part of the NAHase is the cartilage itself via the chondrocytes. A prospective study on early RA patients is now needed to show whether NAHase activity is really a prognostic marker of cartilage degradation in RA.

Acknowledgments

The English text was edited by Dr. Owen Parkes.

References

1. HARRIS ED JR: Clinical features of rheumatoid arthritis. *In* KELLEY WN, HARRIS ED, RUDDY S and SLEDGE CB (Eds.): *Textbook of Rheumatology*. Vol. 1, Philadelphia, W.B. Saunders Co., 1993; 874-911.
2. PINCUS T, CALLAHAN LF: What is the natural history of rheumatoid arthritis ? *Rheum Dis Clin North Am* 1993; 19: 123-51.
3. EMERY P, SALMON M: Early rheumatoid arthritis: Time to aim for remission? *Ann Rheum Dis* 1995; 54 : 944-7.
4. BROWN GM, HUCKERBY TN, ABRAM BL, NIEDUSZYNSKI IA: Characterization of a non-reducing terminal fragment from bovine articular cartilage keratan sulphates containing alpha(2-3)-linked sialic acid and alpha(1-3)-linked fucose. A sulphated variant of the VIM-2 epitope. *Biochem J* 1996; 319: 137-41.
5. NEUFELD EF: Natural history and inherited disorders of a lysosomal enzyme, β-hexosaminidase. *J Biol Chem* 1989; 264: 10927-30.
6. ARNETT FC, EDWORTHY SM, BLOCH DA *et al*.: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 3315-24.
7. LARSEN A, THOEN J: Hand radiography of 200 patients with rheumatoid arthritis repeated with an interval of one year. *Scand J Rheumatol* 1987; 16: 395-401.
8. JACQUES C, BEREZIAT G, HUMBERT L *et al*.: Post-transcriptional effect of IGF-I on IL-1 beta-induced type II secreted phospholipase A2 gene expression in rabbit articular chondrocytes. *J Clin Invest* 1997; 99: 1864-72.
9. CAWSTON TE, MERCER E, DE SILVA M, HAZLEMAN BL: Metalloproteinases and collagenase inhibitors in rheumatoid synovial fluid. *Arthritis Rheum* 1984; 27: 285-90.
10. WOYNAROWSKA B, WIKIEL H, BERNACKI RJ: Human ovarian carcinoma beta-N-acetylglucosaminidase isoenzymes and their role in extracellular matrix degradation. *Cancer Res* 1989; 20: 5598-604.
11. HARRIS PA, STEPHENS RW, GHOSH P, TAYLOR TK: Endo- and exoglycosidases in an experimental rat osteosarcoma. *Aust J Exp Biol Med Sci* 1977; 4: 363-70.
12. VERBRUGGEN G, VEYS EM: N-acetyl-beta-D-hexosaminidase system in synovial fluid. *Scand J Rheumatol* 1976; 5: 39-46.
13. HORSCH M, MAYER C, SENNHAUSER U, RAST DM: Beta-N-acetylhexosaminidase: A target for the design of antifungal agents. *Pharmacol Ther* 1997; 76: 187-218.
14. NURCOMBE HL, BUCKNALL RC, EDWARDS SW: Neutrophils isolated from the synovial fluid of patients with rheumatoid arthritis: Priming and activation *in vivo*. *Ann Rheum Dis* 1991; 50: 147-53.