

# Measurement of plasma concentrations of polymorphonuclear elastase- $\alpha_1$ proteinase inhibitor (elastase- $\alpha_1$ antitrypsin) in patients with rheumatoid arthritis: interference by rheumatoid factor

Rosamonde E Banks, Stuart W Evans, Keith F Taylor, Howard A Bird, John T Whicher

## Abstract

The plasma concentration of granulocyte elastase in complex with  $\alpha_1$  proteinase inhibitor was determined in 32 patients with rheumatoid arthritis and eight with seronegative spondylarthritis complicated by peripheral joint synovitis. Most patients had concentrations of complex which were within the range of the control group when measured by an 'in-house' enzyme linked immunosorbent assay, though several of the patients with rheumatoid arthritis had raised concentrations. When the complexes were measured by a commercially available assay, however, much higher values were obtained for many of the patients with rheumatoid arthritis. Evidence is presented indicating that the commercially available assay may suffer seriously from interference by rheumatoid factor. The possibility is discussed that most patients with rheumatoid arthritis may have plasma concentrations of elastase- $\alpha_1$ PI which are normal or only slightly raised, and that previously published reports using assay systems similar to that available commercially might also have produced falsely raised values for many individuals with rheumatoid arthritis.

Elastase, a neutral proteinase stored within the primary (azurophilic) granules of polymorphonuclear granulocytes, is released in large quantities in inflammation. The biological substrates of elastase include elastin, types III and IV collagen, proteoglycans, and fibronectin.<sup>1</sup>

Circulating elastase is normally found associated with endogenous inhibitors (the major inhibitor is a  $\alpha_1$  proteinase inhibitor ( $\alpha_1$  antitrypsin)) with the remainder being bound to  $\alpha_2$  macroglobulin.<sup>2</sup> Even in chronic inflammatory disorders such as rheumatoid arthritis the circulating inhibitors occur in such excess<sup>3,4</sup> that free elastase activity has been found in the synovial fluid of only one or two patients with extremely high granulocyte counts.<sup>5</sup> To account for possible elastase mediated tissue damage in rheumatoid arthritis it has been proposed that local imbalances of proteinase and proteinase inhibitor must occur within the joint, possibly due to either inactivation of  $\alpha_1$  proteinase inhibitor by phagocyte generated oxidants<sup>6</sup> or to the interference of the cartilage surface with elastase- $\alpha_1$  proteinase inhibitor (elastase- $\alpha_1$ PI) complex formation.<sup>7,8</sup> In addition, the elastase- $\alpha_1$ PI complex itself has been shown to alter the glycosaminoglycan metabolism of synovial cell cultures, possibly via prostaglandin secretion, in a similar way to the altered metabolism seen in chronically inflamed joints.<sup>9</sup>

Elastase- $\alpha_1$ PI has been measured in plasma or synovial fluid samples from patients with rheumatoid arthritis as an indication of polymorphonuclear activation and elastase release<sup>4,5,10-12</sup> and has been proposed as a prognostic marker which is directly related to inflammatory activity and may thus reflect disease activity within the joint. In patients with rheumatoid arthritis very high concentrations of complex have been found in synovial fluid and concentrations significantly in excess of those of controls were found in the plasma of most patients. We extend the previous studies and investigate the possibility that a commercially available assay kit for elastase- $\alpha_1$ PI may produce falsely raised values owing to interference by rheumatoid factor. Elastase- $\alpha_1$ PI concentrations in plasma samples from 40 patients with either rheumatoid arthritis or seronegative spondylarthritis with peripheral joint synovitis were measured both by the commercially available assay kit (Merck) and an enzyme linked immunosorbent assay (ELISA) developed 'in house' and the results compared. The implications of the findings, in particular their correlation with rheumatoid factor levels, are discussed.

## Patients and methods

### PATIENTS

Thirty two outpatients (23 women, nine men) with rheumatoid arthritis and eight patients (four women, four men) with seronegative spondylarthritis and peripheral joint synovitis, categorised according to American Rheumatism Association criteria,<sup>13</sup> were included in the study. The mean ages (ranges) of the two groups were 53 (20-77) years and 54 (30-74) years respectively. The patients ranged from those with recently diagnosed illness to those with longstanding arthritis and were receiving a variety of drugs. The control group consisted of 12 healthy laboratory workers (eight men, four women) with a mean age (range) of 31 (22-45) years.

### MATERIALS

Unless otherwise stated, materials were purchased from Sigma, Dorset.

### METHODS

#### Sample preparation

Venous blood samples from patients and controls were drawn into EDTA coated tubes. Within one to two hours of collection samples were centrifuged at 1500 g (4°C) for 10 minutes, the

Department of Chemical Pathology, University of Leeds, Leeds  
R E Banks  
S W Evans  
J T Whicher

Department of Immunology, General Infirmary at Leeds, Leeds  
K F Taylor

Rheumatism Research Unit, Department of Medicine, General Infirmary at Leeds, Leeds  
H A Bird

Correspondence to:  
Dr Rosamonde E Banks,  
Department of Chemical Pathology, The Old Medical School, University of Leeds, Leeds LS2 9JT.

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plasma removed and stored frozen at  $-40^{\circ}\text{C}$  until assayed for elastase- $\alpha_1$ PI concentrations. Serum samples were also collected and stored at  $-40^{\circ}\text{C}$  until assayed for rheumatoid factor.

#### Commercial elastase- $\alpha_1$ PI assay

Samples were assayed in duplicate by the commercially available Merck PMN elastase immunoassay<sup>14 15</sup> (BDH, Dorset), according to the instructions provided. This assay uses plastic tubes coated with antibodies to polymorphonuclear elastase. The complex present in the sample binds to these antibodies through its elastase component and is detected with an alkaline phosphatase labelled antibody directed at the  $\alpha_1$ PI component.

#### In-house elastase- $\alpha_1$ PI assay

The samples were also assayed in triplicate by an ELISA developed in house as follows. Nunc Immunoplate I microwell plates (Gibco BRL, Paisley) were coated overnight at  $4^{\circ}\text{C}$  with sheep antihuman elastase (Serotec, Oxford) in phosphate buffered saline pH 7.2. Plasma samples were prediluted 50 times with dilution/wash buffer (phosphate buffered saline containing 0.5 M NaCl, 0.1% Tween 20, pH 7.2) and incubated in the coated microwells at room temperature. After two hours the plates were washed three times with dilution/wash buffer, incubated with rabbit antihuman  $\alpha_1$  antitrypsin (Dako, Bucks) for one hour at room temperature, and washed a further three times. Horseradish peroxidase labelled swine antirabbit immunoglobulin (affinity purified; Dako, Bucks) was then added to each well and the plates incubated for a further hour at room temperature. After a further three washes substrate solution (0.1 M citric acid-phosphate buffer, pH 5.0, containing 8 mg *o*-phenylenediamine and 5  $\mu\text{l}$  of 30%  $\text{H}_2\text{O}_2$  per 12 ml of buffer) was added to each well and the plate incubated for 15 minutes in the dark. The reaction was stopped by the addition of 1 M  $\text{H}_2\text{SO}_4$ . Absorbance was read at 492 nm on a Titertek Multiskan MCC plate reader. The concentration of elastase- $\alpha_1$ PI in the samples was calculated from a standard curve prepared using the elastase- $\alpha_1$ PI standards (1–12  $\mu\text{g/l}$ ) provided with the commercial assay.

The working range of the in-house assay was 1  $\mu\text{g/l}$  to 10  $\mu\text{g/l}$ , which is equivalent to 0.1 ng to 1 ng per well with an applied diluted sample volume of 100  $\mu\text{l}$ . Precision within runs (CV) was 4.5% ( $n=10$ , three sample concentrations) and between runs 4.9% ( $n=3$  on six separate runs of a pooled normal plasma sample). The normal reference range found in this study using the in-house assay was 98.9 (29.4)  $\mu\text{g/l}$  (mean (SD),  $n=12$ ). The curves obtained with serial dilutions of various pathological plasma samples were parallel to the calibration curve.

#### Rheumatoid factor levels

Samples were assayed by the sheep cell agglutination test (SCAT; commonly referred to as the Rose-Waaler test),<sup>16 17</sup> which is routinely used clinically within the department of immunology. Results were quantified against serial dilutions of an in-house calibrant stan-

dardised against a WHO reference preparation (1st British standard for rheumatoid arthritis 64/2) and expressed as IU/ml. In addition, some samples were also later assayed for rheumatoid factor by the commercially available Melisa Rheumatoid HAI immunoassay (Walker Laboratories, Cambridgeshire), which was also standardised against a WHO reference preparation.

#### Fast protein liquid chromatography

Six stored serum samples with high rheumatoid factor levels were subjected to fast protein liquid chromatography in order to separate rheumatoid factor from elastase- $\alpha_1$ PI. Fast protein liquid chromatography was performed using a Pharmacia system with a Superose 12 HR 10/30 column, which separates substances on the basis of size. The column was calibrated with a Pharmacia gel filtration calibration kit (Pharmacia, Sweden). The eluent was phosphate buffered saline at a flow of 0.5 ml/min; 500  $\mu\text{l}$  of sample was injected and 500  $\mu\text{l}$  fractions were collected. Fractions were assayed for elastase- $\alpha_1$ PI by both the in-house and Merck assays and, in addition, rheumatoid factor was measured by either the SCAT method or the Melisa assay.

#### STATISTICAL ANALYSIS

Results obtained with the two assay methods were analysed using Wilcoxon's signed rank sum test for paired data.

#### Results

Figure 1 illustrates the plasma elastase- $\alpha_1$ PI concentrations in healthy controls and patients with either rheumatoid arthritis or seronegative

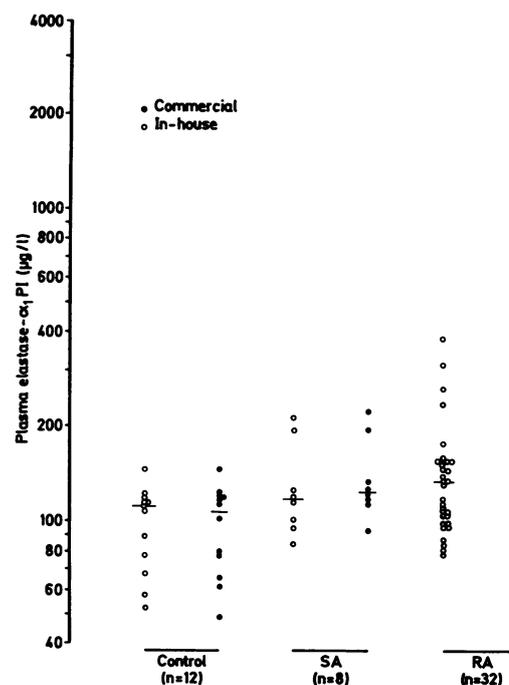


Figure 1: Plasma concentrations of elastase- $\alpha_1$  proteinase inhibitor (elastase- $\alpha_1$ PI) in healthy controls and patients with either seronegative spondylarthritis with peripheral joint synovitis (SA) or rheumatoid arthritis (RA). Results are shown for both the commercial (Merck) assay and the in-house assay. The bar (—) indicates the median value within each group.

spondylarthritis with peripheral joint synovitis. Within each group the individual values obtained using both the commercial (Merck) assay and the in-house assay are shown. There was no significant difference between the two assay methods for values obtained from either the control group or the group with seronegative spondylarthritis. There was a significant difference ( $p < 0.0001$ ), however, between the two assay methods for values obtained from the group with rheumatoid arthritis, with most patients having higher elastase- $\alpha_1$ PI concentrations when measured by the commercial assay than by the in-house assay (in one extreme case 3238.5  $\mu\text{g/l}$  compared with 310  $\mu\text{g/l}$ ).

When the plasma elastase- $\alpha_1$ PI concentrations of the 32 rheumatoid patients were compared with the rheumatoid factor levels (measured by the SCAT method) a significant correlation ( $r = 0.985$ ,  $p < 0.001$ ) was found between rheumatoid factor level and the difference in elastase- $\alpha_1$ PI concentrations between the two assay methods (fig 2). A similar significant correlation was obtained ( $r = 0.858$ ,  $p < 0.001$ ) when rheumatoid factor levels of 22 of the samples measured by the Melisa assay were used (compared with  $r = 0.810$ ,  $p < 0.001$  for the same 22 samples if rheumatoid factor was measured by the SCAT technique).

To clarify the possible involvement of rheumatoid factor in accounting for the difference in values obtained by the in-house and commercial assays the fractions obtained from the fast protein liquid chromatography fractionation of six stored serum samples were assayed for elastase- $\alpha_1$ PI and for rheumatoid factor. Figure 3 shows the results of a representative sample. The initial sample had a rheumatoid factor level of 630 IU/ml and an elastase- $\alpha_1$ PI concentration of 343.3  $\mu\text{g/l}$  (in-house) and 1254.4  $\mu\text{g/l}$  (commercial)—that is, a difference of 911.1  $\mu\text{g/l}$ . When individual fractions were assayed for elastase- $\alpha_1$ PI by the in-house method, one peak was found with a maximum 73.3  $\mu\text{g/l}$  in fraction 18. This peak, when corrected for appropriate dilutions, corresponded to a recovery of  $>90\%$  of the elastase- $\alpha_1$ PI in the initial injected sample

Figure 2: Correlation between rheumatoid factor level (measured by sheep cell agglutination test) and the difference in elastase- $\alpha_1$  proteinase inhibitor (elastase- $\alpha_1$ PI) concentrations obtained between the two assay methods (commercial minus in house) for the 32 patients with rheumatoid arthritis ( $r = 0.985$ ,  $p < 0.001$ ). A rheumatoid factor level of  $< 12.5$  IU/ml is seronegative.

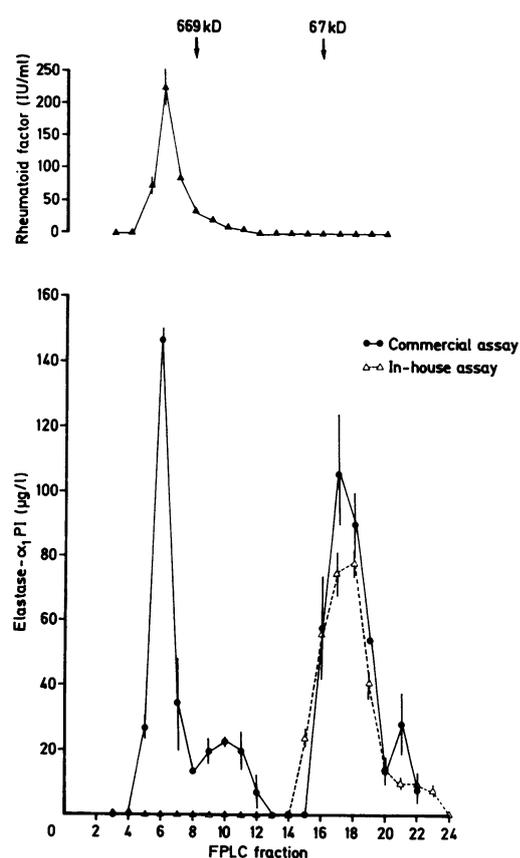
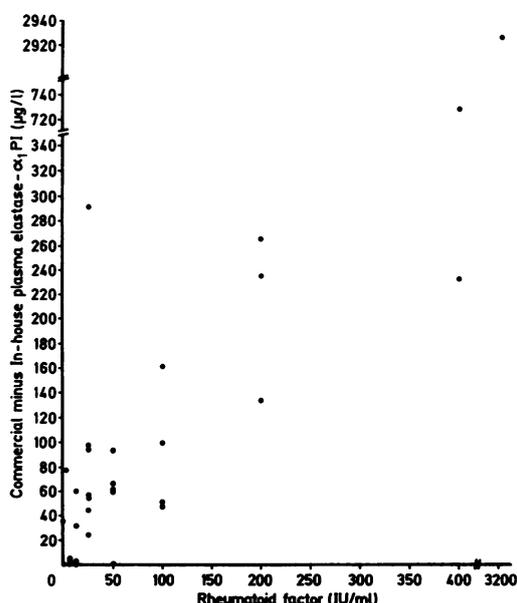


Figure 3: Elastase- $\alpha_1$  proteinase inhibitor (elastase- $\alpha_1$ PI) concentrations of fast protein liquid chromatography (FPLC) fractions from a serum sample with a rheumatoid factor level of 630 IU/ml, showing the different elution profiles obtained for the in-house and commercial methods. Rheumatoid factor levels (measured using Melisa) are shown in IU/ml. Fast protein liquid chromatography was performed as described in the 'Methods' section. The elution position of two molecular weight markers is shown.

(343.3  $\mu\text{g/l}$ ). With the commercial assay method a similar peak was obtained but, in addition, a larger peak was found in fractions 5–8 with a maximum of 143.3  $\mu\text{g/l}$  in fraction 6. This peak represented a recovery of approximately 32% of the 911.1  $\mu\text{g/l}$  difference found between the two assay methods for the initial sample and coincided with a peak in rheumatoid factor levels. Significantly, the recovery of rheumatoid factor in this peak was incomplete with approximately 75% of the rheumatoid factor in the original injected sample being recovered. In addition, aggregation of the fractions containing rheumatoid factor occurred during storage before complex concentrations were determined. This would probably result in substantially less rheumatoid factor than indicated being present in the fractions at the time the concentrations of elastase- $\alpha_1$ PI were determined, and hence less interference with the assay than expected.

## Discussion

The most likely explanation for the discrepancy between the two assay methods is the interference by rheumatoid factor in the commercially available Merck PMN elastase assay. Although it is possible that some factor other than rheumatoid factor is the interfering substance, this is unlikely as it must meet the criteria of

eluting at the same time as the rheumatoid factor in fast protein liquid chromatography and must parallel the rheumatoid factor level in the patients with rheumatoid arthritis in the study. Interference of rheumatoid factor in solid phase immunoassay by its interaction with the Fc portion of IgG leading to exaggerated results is well reported and can be avoided by the use of Fab fragments of immunoglobulins<sup>18</sup> or by adding aggregated IgG to the sample.<sup>19</sup> The reason for the relative lack of interference of rheumatoid factor in the in-house assay, which is similar in principle to the Merck assay but has an additional antibody stage, is not clear but may be due to differences in antibodies, antibody concentrations, or buffer composition. Whether or not values for synovial fluid are also raised when determined by the commercial assay has not been investigated. Although much higher dilutions are used, which may minimise this possibility, rheumatoid factor levels are generally higher in synovial fluid than in plasma.

Figure 2 shows some inconsistencies, with one or two samples having low rheumatoid factor levels and yet quite large discrepancies between the values obtained with the two assay methods. This may be explained if those samples contain IgG rheumatoid factor, which would not be measured by the SCAT technique but which might interfere with the assay. Similarly, the large variation in the discrepancies between the two assay methods for samples with ostensibly the same level of rheumatoid factor is likely to be due to the fact that the rheumatoid factor measurement (SCAT) is semiquantitative, being based on serial doubling dilutions with the levels calculated from the last dilution at which agglutination occurred. Thus a sample reported as 200 IU/ml may theoretically be 200–399 IU/ml. As expected, the correlation between rheumatoid factor and the difference in concentrations of elastase- $\alpha_1$ PI between the commercial and in-house assays was slightly greater when rheumatoid factor was measured using the more accurate Melisa method, though unfortunately this comparison involved only 22 of the samples.

In this study we have shown with an in-house assay that most of the patients with rheumatoid arthritis examined have normal plasma elastase- $\alpha_1$ PI concentrations. This is in marked contrast with previous studies, in which concentrations significantly higher than normal were found in most patients with rheumatoid arthritis.<sup>5 10–12</sup> The small number and lack of controls matched for age and sex in this preliminary study are not likely to account for the difference as our normal range is similar to those reported previously and, also, a lack of dependency of elastase- $\alpha_1$ PI concentrations on age and sex has been reported.<sup>12</sup> Similarly, the patients in this study reflected a range of disease activity and duration as in previous studies.

Whether or not plasma elastase- $\alpha_1$ PI will be of prognostic use must now be further evaluated in the light of these findings and a large longitudinal study is currently in progress to consider this problem. The effects of drug treatment on elastase- $\alpha_1$ PI concentrations will also be examined, though previous reports have

indicated a decrease in concentrations with antirheumatic treatment.<sup>5 12</sup> Depending upon the assay method used, however, such a decrease in elastase- $\alpha_1$ PI concentrations may actually be due partly or totally to a decrease in rheumatoid factor levels, which has been reported to occur with some types of antirheumatic treatment.<sup>20 21</sup>

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