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Proteinase inhibitors in rheumatoid arthritis

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Brackertz, D., Hagmann, J., and Kueppers, F. (1975). *Annals of the Rheumatic Diseases*, **34**, 225–230. **Proteinase inhibitors in rheumatoid arthritis.** The concentrations of five normally occurring protease inhibitors in serum and synovial fluid were compared in patients with rheumatoid arthritis, osteoarthrosis, and normal controls. The patients with rheumatoid arthritis showed a significant rise in alpha$_1$-antitrypsin, alpha$_1$-antichymotrypsin, and inter-alpha-trypsin inhibitor (in decreasing order) in serum as well as in synovial fluid. In synovial fluid the inhibitors were present in their native form and bound to hyaluronate. A large molecular protein with immunological specificity of alpha$_1$-antitrypsin, presumably a complex of alpha$_1$-antitrypsin and a protease, could be shown in synovial fluid of all patients with classical and probable rheumatoid arthritis and not in that of the other subjects studied.

During the past decade it has been possible to subdivide the antiproteolytic activity of human serum into several individual inhibitors with different specificities (Heimburger, Haupt, and Schwick, 1971). Some of these inhibitors belong to the group of acute phase proteins like alpha$_1$-antitrypsin and alpha$_2$-macroglobulin. They are part of a control mechanism of fibrinolysis, coagulation, and the kinin and complement system. It is well known that these systems can release biologically active peptides that have potent effects on blood vessels and on the activity of leucocytes.

The importance of serum proteinase inhibitors is shown by the fact that inborn deficiencies lead to characteristic diseases. Hereditary deficiency of alpha$_1$-antitrypsin is associated with chronic obstructive pulmonary disease (Eriksson, 1965; Laurell and Eriksson, 1963) and severe infantile cirrhosis (Sharp, Bridges, Krivit, and Freier, 1969), while hereditary angioneurotic oedema is caused by low concentration or functional deficiency of complement esterase inhibitor (C1INH) (Donaldson and Evans, 1963; Laurell and Martensson, 1971; Rosen, Charache, Pensky, and Donaldson, 1965).

Antiproteases are probably involved in the protection of tissues against proteolytic enzymes which are released from leucocytes and other cells in various pathological states by limiting their proteolytic activity. It is widely held that proteolytic enzymes in rheumatoid arthritis are responsible for much of the tissue damage in affected joints. Therefore, we investigated the concentrations of several protease inhibitors in serum and in synovial fluids of patients with rheumatoid arthritis and compared them with findings in patients with osteoarthrosis.

Materials and methods

The synovial fluid of patients was obtained by arthrocentesis and immediately centrifuged at +4°C for 15 min at 3000 r.p.m.; the supernate was frozen at −20°C and stored until used. Blood samples were taken from the anticubital vein and the serum was also stored at −20°C. Normal synovial fluid was obtained at autopsy from the knee joints from four subjects without joint disease and serum was obtained from heart blood. These individuals had died from various causes; they had not been dead for more than 6 hours before samples were taken. The following patients were investigated: eighteen (10 males and 8 females) with definite or classical rheumatoid arthritis and six (3 males and 3 females) with probable rheumatoid arthritis according to the criteria of the American Rheumatism Association (Ropes, Bennett, Cobb, Jacox, and Jessar, 1958). Eleven suffered from osteoarthrosis, 7 women and 4 men. They have been under study at the Rheumatic Disease Clinic in Basle for periods ranging from 6 months to 5 years. The individual inhibitors in serum and synovial fluid were measured immunologically according to Mancini, Carbonara, and Heremans (1965). Synovial fluid was treated with 100 IU protease-free hyaluronidase/ml (Batch No. S 110 AB Leo/Helsingborg) for 15 min at 37°C. After this treatment the diffusion coefficient of the individual proteins in synovial fluid was identical to that in serum (Allison

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Table *Summary of data of some clinical findings.*

Clinical group	*No. of cases*	*Synovial fluid*		*Serum*		
		*Total protein (g/100 ml)**	*White blood cells (mm^3)**	*α_2 Globulin relative %**	*ESR (mm)* *1st hour*	*2nd hour**
Classical rheumatoid arthritis	18	4·03 (2·9–5·4)	8750 (2100–26000)	13·1 (11·0–16·0)	36	75
Osteoarthrosis	11	2·80 (2·4–3·8)	1880 (100–7750	6·8 (3·0–10·0)	19	40
Probable rheumatoid arthritis	6	4·00 (3·0–4·8)	6442 (250–1850)	8·4 (3·5–13·0)	40	63

* Average value.
Range in parentheses.

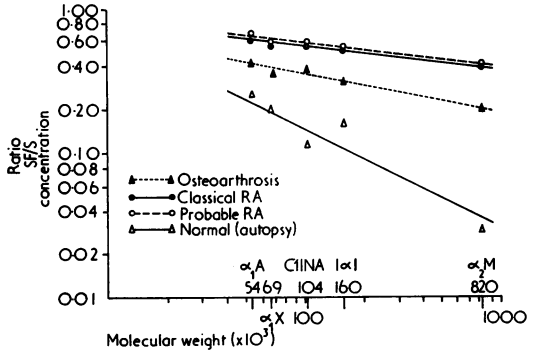

FIG. 1 *Ratio of synovial fluid concentration to serum concentration (SF/S) plotted logarithmically against the molecular weight of 5 proteinase inhibitors in each group of patients. Note the higher SF/S ratios and changes in slope of the lines with increasing degree of synovial inflammation, reflecting increased synovial permeability to serum proteins and proportionately greater increases in permeability to large molecules, respectively. α_1A = alpha$_1$-antitrypsin, α_1X = alpha$_1$-antichymotrypsin, $C\bar{1}INA$ = $C\bar{1}$-inactivator, $I\alpha I$ = inter-alpha-trypsininhibitor, α_2M = alpha$_2$-macroglobulin*

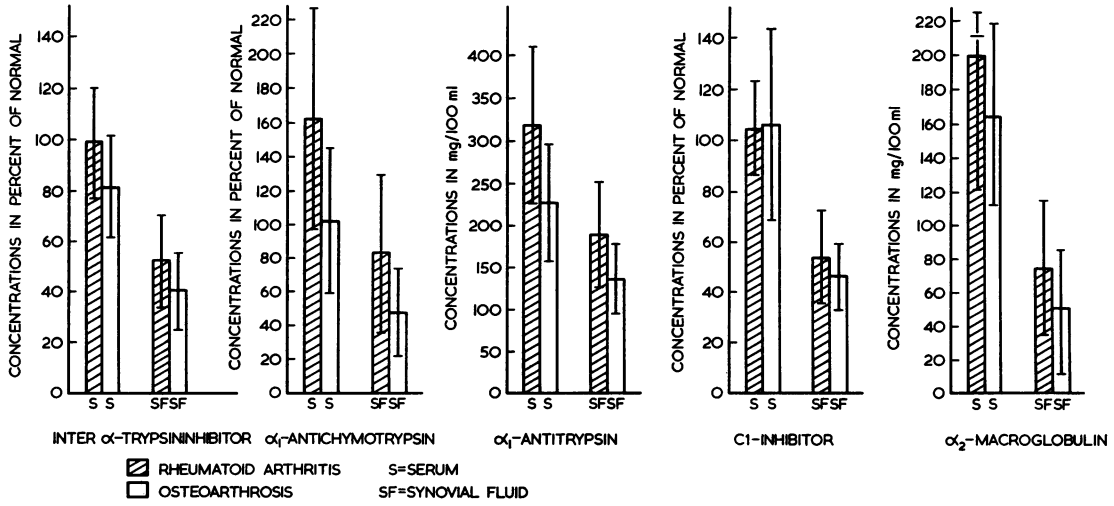

FIG. 2 *Concentrations of proteinase inhibitors in serum and synovial fluid. Note the significant difference in serum and synovial fluid for α_1-antitrypsin, inter-α-trypsininhibitor, and α_1-antichymotrypsin in patients with rheumatoid arthritis in comparison to patients with osteoarthrosis. The difference for $C\bar{1}$-inhibitor and α_2-macroglobulin in serum as well as in synovial fluid was not significant*

and Humphrey, 1960). Normal values were determined from a group of 20 healthy blood donors. Alpha$_1$-antitrypsin and alpha$_2$-macroglobulin were measured with Partigen plates (Op-/Batch No. 01781 and Op-/Batch No. 1903, Behringwerke/Marburg). Human serum was used as standard (Op/Batch No. 972b, Behringwerke/Marburg). Microimmunoelectrophoresis was done by the method of Scheidegger (1955). The double-diffusion experiments were performed according to Ouchterlony (1949). Gel chromatography of synovial fluid was done as follows. A 4 ml sample of synovial fluid from a patient with rheumatoid arthritis was incubated with hyaluronidase (100 IU/ml synovial fluid) for 15 min at 37°C. Then it was applied to a Sephadex G 200 column 2·5 × 95 cm. The eluting buffer was 0·1 mol/l Tris-HCl, 0·1 mol/l NaCl pH 7·6; 3 ml fractions were collected and concentrated fourfold by ultrafiltration.

Results

Some clinical findings that indicate the degree of local and systemic inflammation are listed in the Table. To obtain better information about the severity of synovial inflammation, the ratio of synovial fluid concentration to serum concentration (SF/S) and the molecular weights of the 5 proteinase inhibitors of each group of patients studied was plotted logarithmically according to Kushner and Somerville (1971) (Fig. 1). It is known that the concentration of a given synovial protein relative to its serum concentration reflects quite accurately the degree of synovial permeability and inflammation in the absence of local production or consumption. The resulting regression lines were compared to that of the four control (autopsied) subjects without joint disease (Fig. 1). It can be seen that with an increasing degree of synovial inflammation the slope becomes less steep, reflecting proportionately greater permeability to large molecules. There was a significant difference of the levels in serum and synovial fluid for alpha$_1$-antitrypsin, inter-alpha-trypsin inhibitor, and alpha$_1$-antichymotrypsin in patients with rheumatoid arthritis in comparison to patients with osteoarthrosis (Fig. 2). In serum the difference was most pronounced for alpha$_1$-antitrypsin ($P < 0{\cdot}01$) followed by alpha$_1$-antichymotrypsin ($P < 0{\cdot}02$), and inter-alpha-trypsin inhibitor ($P < 0{\cdot}05$).

Immunoelectrophoresis of native synovial fluid showed the proteinase inhibitors in their characteristic position but always followed by a slower moving antigenically identical component (Fig. 3) which disappeared after incubation with hyaluroni-

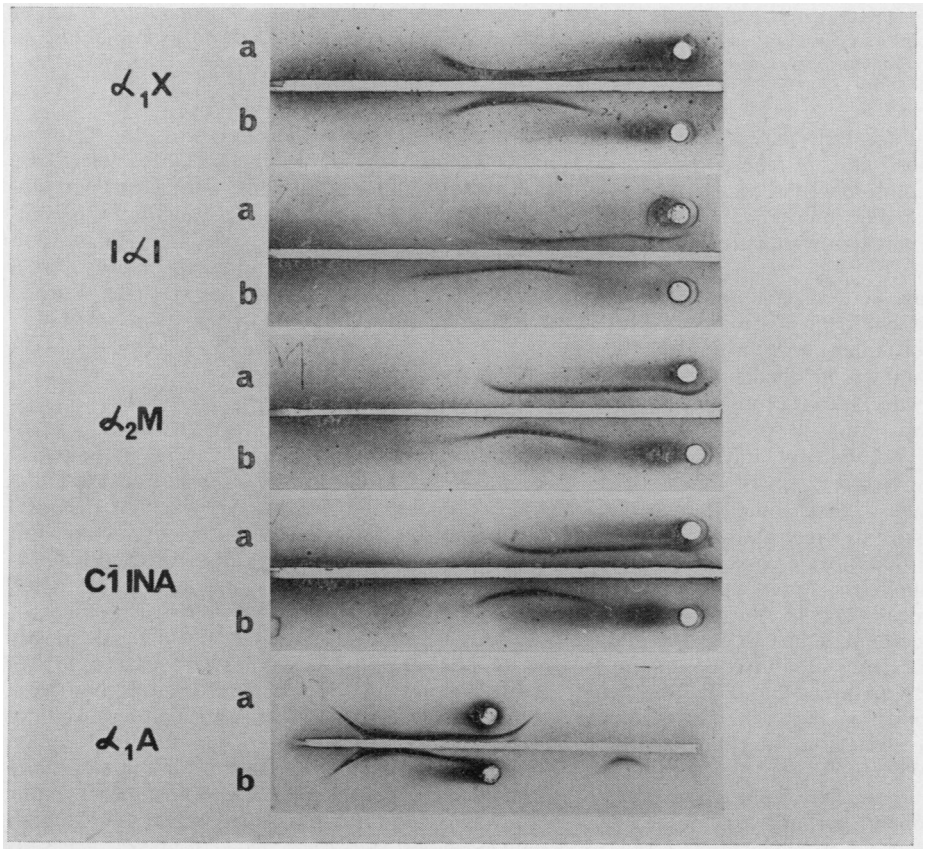

FIG. 3 *Identification of 5 proteinase inhibitors in (a) native and (b) hyaluronidase-treated synovia by immunoelectrophoresis using monospecific antisera. Anode is to the left.* $\alpha_1 X$ = *alpha*$_1$*-antichymotrypsin,* $I\alpha I$ = *inter-alpha-trypsininhibitor,* $\alpha_2 M$ = *alpha*$_2$*-macroglobulin,* $C\bar{1}INA$ = $C\bar{1}$*-inactivator,* $\alpha_1 A$ = *alpha*$_1$*-antitrypsin*

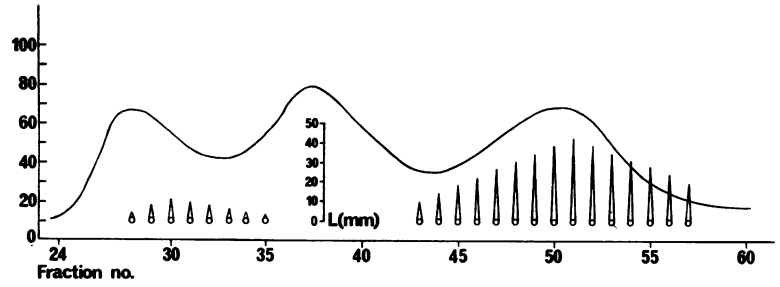

FIG. 4 *Chromatography of synovia on Sephadex G 200 and determination of α_1-antitrypsin fractions by electroimmunodiffusion. Protein concentration in fractions expressed as optical density at 280 nm.*

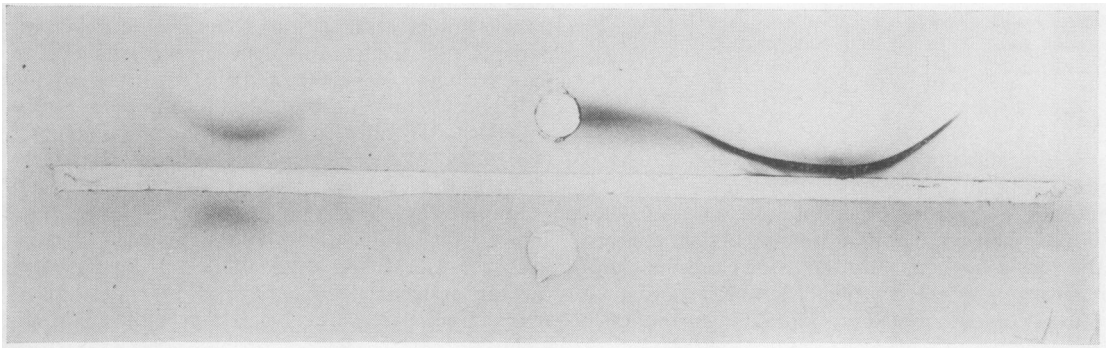

FIG. 5 *Immunoelectrophoresis of synovial fluid from a patient with rheumatoid arthritis after hyaluronidase treatment (upper well) and the pooled and concentrated fractions from Sephadex G 200 of the early eluting material with α_1-antitrypsin specificity (lower well). Trough: antiserum to α_1-antitrypsin. Anode is to the right.*

dase. Except in alpha$_1$-antitrypsin, only the 24 patients with classical and probable rheumatoid arthritis showed, in addition to the characteristic precipitate in the alpha$_1$-region, a new component reacting with the specific antiserum moving towards the cathode.

Further experiments were designed to characterize this component. To exclude the possibility that it represents a complex of alpha$_1$-antitrypsin and hyaluronidase, normal serum was incubated with the same concentration of hyaluronidase and submitted to immunoelectrophoresis using a specific antiserum. There was no change in mobility of alpha$_1$-antitrypsin and no cathodally moving component appeared.

Gel chromatography of hyaluronidase-treated synovial fluid determined by electroimmunodiffusion revealed most of the alpha$_1$-antitrypsin eluting in the third peak, but a definite maximum eluting between the first and second peak (Fig. 4). When the material that eluted from the column in the first maximum was pooled, concentrated, and submitted to immunoelectrophoresis, it was apparent that it had the same electrophoretic mobility as the cathodally moving component in the synovial fluid after hyaluronidase treatment (Fig. 5). We assume that the early eluting material represents a complex of alpha$_1$-antitrypsin with another component, perhaps a protease. This complex is probably identical with the 'alpha$_1$-antitrypsin of high molecular weight' detected by Ganrot (1967) in a number of normal and pathological human sera. The molecular weight of this complex was estimated by the method of Allison and Humphrey (1960) as approximately 200000. In a double diffusion experiment the complex showed antigenic identity with alpha$_1$-antitrypsin.

Discussion

The presence of high concentrations of protease inhibitors in synovial fluid under various pathological conditions is of considerable interest. Because of the difficulty in obtaining normal synovial fluid, the number of controls was small (4 autopsy patients). But the differences between the pathological and the normal synovial fluids are very striking. Kushner and Somerville (1971) have shown that the presence of any serum protein in synovial fluid depends mostly on its Stokes radius. Molecules with a larger Stokes radius are present in lower concentrations than those with a smaller radius. With increasing permeability of the vascular walls and the interstitial hyaluronate during inflammation, this relationship changes: there is an absolute and relative increase in concentrations of proteins with high molecular weight. The highest

absolute increase we have seen in the concentration of alpha$_1$-antitrypsin, alpha$_1$-antichymotrypsin, and inter-alpha-trypsin inhibitor was in serum and synovial fluid of patients with rheumatoid arthritis. The concentrations of these proteins are also significantly higher in comparison to patients with osteoarthrosis. This finding is in agreement with the known fact that alpha$_1$-antitrypsin and alpha$_1$-antichymotrypsin (Aronsen, Ekelund, Kindmark and Laurell, 1972; Kueppers, 1968) belong to the group of acute phase proteins that respond to inflammatory stimuli with a substantial rise in concentration.

As Sandson and Hamerman (1964) have shown, the inter-alpha-trypsin inhibitor is present in high concentrations in synovial fluid and forms a complex with hyaluronate in patients with rheumatoid arthritis. From our observations this feature is not unique for the inter-alpha-trypsin inhibitor, but we have also seen complex formation of hyaluronate and the 4 other proteinase inhibitors tested (Fig. 3). Alpha-$_1$ antitrypsin differs from the other inhibitors by the fact that the native protein and the high molecular weight complex is bound to hyaluronate. The molecular weight as judged from the elution pattern from a Sephadex G 200 column and double diffusion (Allison and Humphrey, 1960) is approximately 200000. Some preliminary measurements of synovial fluid from patients with rheumatoid arthritis show a lower protease inhibiting activity than one might expect from the concentration of these inhibitors found immunologically (D. Brackertz and F. Kueppers, unpublished, 1973). This finding could be explained by the inclusion of inactive inhibitor complexes in the immunological determinations.

It seems that the presence of protease inhibitors at the site of inflammation, first shown by Opie (Opie and Barker, 1907), is important in limiting the destructive activity of proteases that are liberated from polymorphonuclear leucocytes and other necrotizing tissues. At least two such enzymes have been isolated and characterized (Janoff and Scherer, 1968; Lazarus, Daniels, Brown, Bladen, and Fullmer, 1968). It would be interesting to know which proteases are present as inhibitor complexes and which remain uninhibited; for proteases that are not inactivated should be potentially more destructive ones. The inhibition of these proteases by other normally occurring inhibitors as well as synthetic ones could be an important step in preventing tissue damage in rheumatoid arthritis.

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